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(54) Title: MONOGENOUS PREPARATIONS OF CYTOTOXIC CONJUGATES

(57) Abstract

Monogenous preparations of conjugates of fibroblast growth factors (FGF) with cytotoxic agents are provided. Substantially all of the cytotoxic conjugates in the resulting monogenous preparation contain the same molar ratio of cytotoxic agent to polypeptide reactive with an FGF receptor. The conjugates are produced by chemical conjugation and also by expression of DNA encoding the conjugate to produce fusion proteins. The chemical conjugates are prepared by reacting FGF, which has at least one of the reactive cysteines replaced or deleted, with purified mono-derivatized saporin or with saporin that has been modified at or near one terminus by addition of a cysteine residue or replacement of a residue with cysteine. The resulting preparations are substantially monogenous. Fusion proteins are produced by expressing DNA constructs encoding muteins of an FGF in which one or more of any reactive cysteines have been deleted or replaced linked to DNA encoding a cytotoxic agent are also provided. The DNA constructs are expressed in host cells to produce monogenous preparations of cytotoxic FGF conjugates. In embodiments in which the FGF portion of the conjugate has been modified so that there are no cysteines available for reaction, the resulting compositions are free of aggregates. The monogenous preparations of conjugates provided herein are potent cytocidal agent to cells bearing the FGF receptor.

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MONOGENOUS PREPARATIONS OF CYTOTOXIC CONJUGATES

FIELD OF THE INVENTION

This invention is related to the preparation and use of cytotoxic conjugates. In particular, substantially monogenous preparations of cytotoxic conjugates, homogeneous compositions of cytotoxic conjugates and methods for preparing such cytotoxic conjugates are provided.

BACKGROUND OF THE INVENTION

One goal in pharmacology is to design specific agents that act with high specific activity only on targeted cells or tissues. This aim is of particular importance, for example, in the design of agents for treatments of diseases, such as neoplastic disease and diseases of viral origin, in which the ratio of toxic dose to therapeutic dose is very low and the dosage must be minimized. Numerous approaches to achieving this goal have been developed. Among these are the use of agents, such as growth factors, that act only on specific cells, and the use of toxins that are relatively non-toxic unless delivered intracellularly.

Fibroblast growth factors and fibroblast growth factor receptors

During the last twenty-five years, a great deal of attention has been directed towards the identification and characterization of factors that stimulate the growth, proliferation and differentiation of specific cell types. Numerous growth factors and families of growth factors that share structural and functional features have been identified. Many of these factors have multifunctional activities and affect a wide spectrum of cell types.

One family of growth factors that has a broad spectrum of activities is the fibroblast growth factor (FGF) family. This family of proteins includes FGFs designated FGF-1 through FGF-9 (or acidic FGF (aFGF), basic FGF (bFGF), int-2, hst-1/K-FGF, FGF-5, FGF-6/Hst-2, keratinocyte growth factor (KGF), FGF-8 and FGF-9, respectively). These proteins share the ability to bind to heparin, induce intracellular receptor-mediated tyrosine

phosphorylation and the expression of the c-fos mRNA transcript, and stimulate DNA synthesis and cell proliferation.

Acidic and basic FGF, which were the first members of the FGF family that were characterized, are about 55% identical at the amino acid level and are highly conserved among species. Basic FGF has a molecular weight of approximately 16 kD, is acidic and temperature sensitive and has a high isoelectric point. Acidic FGF has an acidic isoelectric point. The other members of the FGF family have subsequently been identified on the basis of amino acid sequence homologies with aFGF and bFGF and common physical and biological properties, including the ability to bind to one or more FGF receptors. Basic FGF, int-2, hst-1/K-FGF, FGF-5, hst-2/FGF-6 and FGF-8 are oncogenes. For example, bFGF is expressed in melanomas, int-2 is expressed in mammary tumor virus and hst-1/K-FGF is expressed in angiogenic tumors. Acidic FGF, bFGF, KGF and FGF-9 are expressed in normal cells and tissues.

FGFs exhibit a mitogenic effect on a wide variety of mesenchymal, endocrine and neural cells. They are also important in differentiation and development. Of particular interest is their stimulatory effect on collateral vascularization and angiogenesis. Such effects have stimulated considerable interest in FGFs as therapeutic agents, for example, as pharmaceuticals for wound healing, neovascularization, nerve regeneration and cartilage repair. In addition to potentially useful proliferative effects, FGF-induced mitogenic stimulation may, in some instances, be detrimental. For example, cell proliferation and angiogenesis are an integral aspect of tumor growth. Members of the FGF family, including bFGF, are thought to play a pathophysiological role, for example, in tumor development, rheumatoid arthritis, proliferative diabetic retinopathies and other complications of diabetes.

The effects of FGFs are mediated by high affinity receptor tyrosine kinases on the cell surface membranes or FGF-responsive cells (see, e.g., Imamura et al. (1988) Biochem. Biophys. Res. Comm. 155:583-590;

Huang et al. (1986) J. Biol. Chem. 261:9568-9571, which are incorporated herein by reference). Lower affinity receptors also play a role in mediating FGF activities. The high affinity receptor proteins, which are single chain polypeptides with molecular weights ranging from 110 to 150 kD, depending on cell type, constitute a family of structurally related FGF receptors. Four FGF receptor genes have been identified, and, at least two of these genes generate multiple mRNA transcripts via alternative splicing of the primary transcript.

Ribosome-inactivating proteins

Ribosome-inactivating-proteins (RIPs), which include ricin, abrin and saporin, are plant proteins that catalytically inactivate eukaryotic ribosomes. Some RIPs, such as the toxins abrin and ricin, contain two constituent chains: a cell-binding chain that mediates binding to cell surface receptors and internalizing the molecule; and a chain responsible for toxicity. Such RIPs are type II RIPs. Single chain RIPs, such as the saporins, do not have a cell-binding chain. As a result, unless internalized, they are substantially less toxic to whole cells than the RIPs that have two chains.

RIPS inactivate ribosomes by interfering with the protein elongation step of protein synthesis. For example, the RIP saporin (hereinafter also referred to as SAP) has been shown to inactivate 60S ribosomes by cleavage of the n-glycosidic bond of the adenine at position 4324 in the rat 28S ribosomal RNA (rRNA). The particular region in which A^{4324} is located in the rRNA is highly conserved among prokaryotes and eukaryotes. A^{4324} in 28S rRNA corresponds to A^{2660} in Escherichia coli (E. coli) 23S rRNA. Several RIP's also appear to interfere with protein synthesis in prokaryotes, such as E. coli.

Several structurally related RIP's have been isolated from seeds and leaves of the plant Saponaria officinalis (soapwort). Among these, SAP-6 is the most active and abundant, representing 7% of total seed proteins. Saporin is very stable, has a high isoelectric point, does not contain

carbohydrates, and is resistant to denaturing agents, such as sodium dodecyl sulfate (SDS), and a variety of proteases. The amino acid sequences of several saporin-6 isoforms from seeds are known and there appear to be families of saporin RIPs differing in few amino acid residues. Because saporin is a type I RIP, it does not possess a cell-binding chain. Consequently, its toxicity to whole cells is much lower than other toxins, such as ricin and abrin. When internalized by eukaryotic cells, however, its cytotoxicity is 100- to 1000-fold more potent than ricin A chain.

Cytotoxic conjugates

Cytotoxins, such as saporin and ricin A chain, have been covalently linked to cell surface binding proteins to produce cytotoxic chemical conjugates or have been linked to antibodies to produce immunotoxins that are targeted to, and internalized by, specific cells. For example, basic fibroblast growth factor (bFGF) has been chemically conjugated to saporin-6 to produce the mitotoxin bFGF-SAP (see, e.g., U.S. Patent No. 5,191,067 to Lappi et al.; and Lappi et al. (1989) Biochem. and Biophys. Res. Comm. 160:917-923). The resulting FGF-SAP conjugates have been used to treat restenosis (see, e.g., International Patent Application No. WO 92/11872, which is based on U.S. Application Serial No. 07/637,074; see, also U.S. Patent No. 5,308,622) and other FGF-mediated disorders. Treatment is effected by local or intravenous administration of a therapeutically effective amount of the FGF conjugate following, for example, balloon angioplasty. Basic FGF-SAP conjugates also have shown promise as agents for the treatment of certain tumors. The growth of melanomas and other tumors that express receptors to which FGFs bind can be inhibited by FGF-SAP (see, e.g., published International Application WO 92/04918, which is based on U.S. Application Serial No. 07/585,319, filed 9/19/90; published International Application No. WO 92/04918, which is based on U.S. Patent Application Serial No. 07/585,319; and Beitz et al. (1992) Cancer Research 52:227-230).

Conjugates are often synthesized by the use of reactive sulfhydryls either found naturally, as in the case of ricin A chain, in the cytotoxic moiety and the targeting moiety. If not present, sulfhydryls are introduced into the cytotoxic agent using a chemical coupling agent so that conjugation is possible for antibodies and for RIPs, such as SAP, that are devoid of native or available sulfhydryls. The chemistry of conjugation, however, gives rise to various structures, resulting in a heterogeneous population of products that are difficult to separate from each other. These structures can include conjugates containing more than one RIP attached to the targeting moiety, more than one targeting moiety attached to the RIP, or more than one RIP attached to more than one targeting moiety. The resulting structures also form aggregates because of interactions among the conjugates, particularly among free sulfhydryls in the conjugates. Because of the difficulties encountered in separating the resulting conjugates with different structures, heterogeneous mixtures are often used in experiments and even therapeutic applications.

For example, bFGF is conjugated via a cysteine residue to saporin, which is first derivatized with N-succinimdyl-3(2-pyridyldithio)propionate (SPDP). Basic FGF has at least two cysteines available for reaction with SPDP-derivatized saporin. Consequently, reaction of the bFGF with the SPDP-derivatized SAP results in an array of molecules, which probably differ with respect to biologically relevant properties and may not be ideal for <u>in vivo</u> applications. Gel electrophoresis and western blotting verify that a number of higher molecular weight species are formed. The species contain SAP to FGF ratios of 0.5, 1, 2 and other oligomeric combinations. There is very little information on the relative activities of the various constituents of the heterogeneous population, though it has been reported that polymeric RIPs have increased non-specific toxicities.

To develop FGF-SAP and other cytotoxic agents into acceptable pharmaceutical agents for treating deleterious disease states, it would be desirable to have a monogenous molecule that is well-characterized

physically, chemically and biologically. Therefore, it is an object herein to provide methods for the production of monogenous preparations of cytotoxic FGF conjugates and of homogeneous compositions containing conjugates of FGF and cytotoxins. It is also an object herein to provide the FGF cytotoxic conjugates that are produced by these methods. It is also an object herein to provide compositions that contain homogeneous populations of FGF cytotoxic conjugates or mixtures of monogenous cytotoxic conjugates.

SUMMARY OF THE INVENTION

Monogenous preparations of cytotoxic conjugates and compositions containing homogeneous (non-aggregated) populations of cytotoxic conjugates are provided. The cytotoxic conjugates contain a polypeptide that is reactive with an FGF receptor (also referred to herein as an FGF protein), such as bFGF, linked to a cytotoxic agent. In a given preparation substantially all of the cytotoxic conjugates have the same ratio of the polypeptide that is reactive with an FGF receptor to cytotoxic agent. In preferred embodiments, the cytotoxic conjugates contain one molecule of FGF protein per molecule of cytotoxic agent.

Polypeptides that are reactive with an FGF receptor (FGF proteins) include any molecule that reacts with FGF receptors on cells that bear FGF receptors and results in internalization of the linked cytotoxic agent. Particularly preferred polypeptides that are reactive with an FGF receptor include members of the FGF family of polypeptides, muteins of these polypetides, and chimeric or hybrid molecules that contain portions of any of these family members, as long as the resulting polypeptide binds to FGF receptors and internalizes a linked cytotoxic agent and the resulting preparation of cytotoxic conjugates that contain the FGF protein is monogenous (i.e. each conjugate in a preparation of such conjugates has th same molar ratio of FGF protein to cytotoxic agent).

The cytotoxic agents include any molecule that, when internalized, is cytotoxic to eukaryotic cells. Such cytotoxic agents include, but are not

limited to, ribosome inactivating proteins, inhibitors of DNA, RNA and/or protein synthesis and other metabolic inhibitors. In certain embodiments, the cytotoxic agent is a ribosome-inactivating protein (RIP), such as, for example, saporin, although other cytotoxic agents can also be advantageously used.

The preparation may be produced by chemical means so that the resulting conjugates are chemical conjugates or using DNA encoding chimeric molecules to produce fusion proteins. The components of the conjugates may also be produced by expression of DNA or by chemical synthesis or any other method known to those of skill in this art.

The conjugate can be represented by formula:

$(FGF)_n$-(cytotoxic agent)$_m$,

with the understanding that the FGF and cytotoxic agent may be linked in any order and through any appropriate linkage, as long as the resulting conjugate binds to an FGF receptor and internalizes the cytotoxic agent(s) in cells bearing an FGF receptor. FGF refers to the polypeptide reactive with an FGF receptor, n and m, which in monogenous preparations are integers, are the same or different, and are 1 to 6, preferably 1 to 4, and typically 1 or 2, and if m or n, or m and n are greater than 1, then the conjugate may contain more than one cytotoxic agent and more than one FGF.

Cytotoxic conjugates that contain a plurality of monomers of an FGF protein linked to the cytotoxic agent are also provided. These conjugates that contain several, typically two to about six, monomers can be produced by linking multiple copies of DNA encoding the FGF fusion protein, typically head-to-tail, under the transcriptional control of a single promoter region.

To produce a monogenous preparation of cytotoxic conjugates or homogeneous compositions of such conjugates, the cytotoxic agent is linked to the polypeptide that is reactive with an FGF receptor by the methods provided herein. Each member of the resulting preparation of cytotoxic conjugate contains the same molar ratio of cytotoxic agent to

polypeptide that is reactive with an FGF receptor. Generally each conjugate contains one molecule of each of the constituents. In addition, in preferred embodiments the resulting conjugates do not form aggregates.

Methods for the preparation of the cytotoxic agent, such as a ribosome inactivating protein (RIP), including, but not limited to, saporin, and the FGF polypeptides and the monogenous preparation of cytotoxic conjugates that contains a defined molar ratio of each of the constituents are provided. These methods include chemical conjugation methods and methods that rely on recombinant production of the cytotoxic conjugates. The methods result in monogenous preparations of cytotoxic conjugates that can be used, in preferred embodiments, to prepare homogeneous compositions of monogenous cytotoxic conjugates.

The chemical method relies on several means to reduce the heterogeneity of the resulting cytotoxic conjugate and to avoid interactions among the conjugates that result in aggregate formation. In preferred embodiments, the FGF portion of the conjugate is treated so that only one cysteine is available for reaction with the cytotoxic agent and the cytotoxic agent, if necessary, is derivatized and only a single species is selected for reaction with the modified FGF. The cytotoxic agent, may also be modified to include a cysteine residue. The locus of the cysteine residue is selected such that the cysteine residue is available for conjugation with the available cysteine in the FGF polypeptide and the resulting conjugate is cytotoxic upon internalization by targeted eukaryotic cells.

In accordance with this embodiment, modified saporin is provided. Such modifications include, but are not limited to, the introduction of a Cys residue at or near the N-terminus. Saporin is modified by addition of a cysteine residue at the N-terminus-encoding portion of the DNA by addition of a Met-Cys. Saporin also has been modified herein by insertion of a cysteine at position 4 or 10 in place of the wild type residue. The resulting saporin can then be reacted with an available cysteine on an FGF to

produce conjugates that are linked via the added Cys or Met-Cys on saporin.

In practicing the chemical method, site-directed mutagenesis has been used to reduce the heterogeneity of the chemical conjugate by replacing one of the reactive cysteines in bFGF with a residue, such as serine, that does not alter the cytotoxicity of the resulting conjugate, and leaves only one cysteine available for reaction with the cytotoxic agent. In preferred embodiments, the cytotoxic agent is a single species of derivatized SAP. Because there are slight charge differences among different derivatized SAP species that are produced upon the derivatization of SAP, it has been found herein that it is possible to isolate substantially pure mono-derivatized SAP. Reaction of mono-derivatized SAP with mono-reactive cysteine basic FGF produces a monogenous preparation of cytotoxic conjugates and homogeneous populations of conjugates that are highly cytotoxic to FGF-receptor-bearing cells. In other embodiments, the saporin is modified at or near the N-terminus to include a cysteine residue, so that the resulting modified saporin can react with the FGF protein without further derivatization.

The recombinant method relies on the expression of DNA that encodes an FGF protein, modified to remove all cysteines that contribute to aggregate formation, linked to DNA encoding the cytotoxic conjugate. DNA encoding the FGF polypeptide is mutagenized so that no cysteines are available in the resulting conjugate for interaction with other conjugates. The DNA encoding the modified FGF protein is linked directly to the DNA encoding the N-terminus of the saporin polypeptide or via one, preferably two, or more codons that encode a linking peptide or amino acid. The number of linking codons is selected such that the resulting DNA encodes a fusion protein that is cytotoxic to selected cells.

The combination of the modified FGF protein and linked cytotoxic agent is prepared as a chimera, using recombinant DNA techniques. The fusion protein molecule is designed and produced in such a way that the

FGF protein portion of the conjugate is available for recognition of its respective cell-surface receptor and can target the conjugate to cells containing its respective cell-surface receptor. In a preferred embodiment, the FGF protein is FGF that has been modified by replacement of the cysteine residues at positions 78 and 96 with serine residues.

The resulting monogenous preparation of conjugates and homogeneous compositions of conjugates produced by any of the methods described herein can be used in pharmaceutical compositions to treat FGF-mediated pathophysiological conditions by specifically targeting to cells having FGF receptors and inhibiting proliferation of or causing death of the cells. Such pathophysiological conditions include, for example, tumor development, restenosis, Dupuytren's Contracture, certain complications of diabetes such as proliferative diabetic retinopathies, and rheumatoid arthritis. The treatment is effected by administering a therapeutically effective amount of the FGF conjugate, for example, in a physiologically acceptable excipient. Additionally, the conjugate can be used to target cytotoxic agents into cells having FGF receptors, and to inhibit the proliferation of such cells.

The resulting preparations of monogenous FGF conjugates or homogeneous compositions of conjugates may also be administered in conjunction with anti-tumor agents, such as cis-platin. Such combination therapy enhances the anti-tumor activity of the FGF-conjugates. In particular, administration of cis-platin in conjunction with an FGF-cytotoxic conjugate enhanced the anti-tumor activity of the FGF-cytotoxic conjugate. In particular, a method for inhibiting the proliferation of tumor cells that bear FGF receptors by administering a proliferation-inhibiting amount of a cytotoxic conjugate and a cytotoxic amount of cis-platin, in which the amounts of each are such that the combination of cytoxic conjugate and cis-platin kills or inhibits the growth of the tumor cells, is provided.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the subject matter herein belongs. All U.S. patents and all publications mentioned herein are incorporated in their entirety by reference thereto.

The amino acids, which occur in the various amino acid sequences appearing herein, are identified according to their well-known, three-letter or one-letter abbreviations. The nucleotides, which occur in the various DNA fragments, are designated with the standard single-letter designations used routinely in the art.

As used herein, cytotoxic agents include saporin, the ricins, abrin and other RIPs, Pseudomonas exotoxin, inhibitors of DNA, RNA or protein synthesis or other metabolic inhibitors that are known to those of skill in this art. Saporin is preferred, but other suitable RIPs include, but are not limited to, ricin, ricin A chain, maize RIP, gelonin, diphtheria toxin, diphtheria toxin A chain, trichosanthin, tritin, pokeweed antiviral protein (PAP), mirabilis antiviral protein (MAP), Dianthins 32 and 30, abrin, monordin, bryodin, shiga and others known to those of skill in this art. The term RIP is used herein to broadly include such cytotoxins, as well as other cytotoxic molecules that inhibit cellular metabolic process, including transcription, translation, biosynthetic or degradative pathways, DNA synthesis and other such process, or that kill cells.

As used herein, saporin (abbreviated herein as SAP) refers to polypeptides having amino acid sequences found in the natural plant host Saponaria officinalis, as well as modified sequences, having amino acid substitutions, deletions, insertions or additions, which still express substantial ribosom -inactivating activity. Purified preparations of saporin are frequently observed to include several molecular isoforms of the protein. It is understood that differences in amino acid sequences can

occur in saporin from different species as well as between saporin molecules from individual organisms of the same species.

As used herein, N-terminal extension, refers to a peptide region that is linked to the amino terminus of a biologically active portion of a saporin polypeptide. As demonstrated herein, when saporin is produced by expressing DNA encoding in a host cell, it is expressed with an N-terminal extension. The N-terminal extension serves to render the saporin polypeptide portion of the saporin-containing protein either nontoxic to the host upon expression of the protein in the host or substantially less toxic to the host than the expression of a saporin polypeptide without an N-terminal extension. N-terminal extensions having as few as 2 amino acids, and up to many amino acids, are provided. The length of the N-terminal extension is not important as long as the resulting cytotoxic conjugate binds to cell surface receptors, internalizes the cytotoxic agent and is cytotoxic upon internalization, can be employed. The precise number for the upper limit can be determined empirically, using cytotoxicity assays, such as those exemplified herein, that are known to those of skill in this art. Presently preferred N-terminal extension regions are on the order of about 2 to 15 amino acids. Most preferred N-terminal extension regions are in the range of about 2 to about 10 amino acids.

As used herein, a modification that is effected substantially near the N-terminus of a cytotoxic agent, such as saporin, is generally effected within the first about ten residues of the protein. Such modifications, include the addition or deletion of residues, such as the addition of a cysteine facilitate conjugation between the polypeptide reactive with an FGF receptor or fragment of the polypeptide and the cytotoxic moiety portion to form cytotoxic agents that contain a defined molar ratio, preferably a ratio of 1:1, of cytotoxic agent and polypeptide reactive with an FGF receptor or fragment of the polypeptide.

As used herein, a mitotoxin is a cytotoxic molecule targeted to specific cells by a mitogen.

As used herein, the term cytotoxic agent refers to a molecule capable of inhibiting cell function. The agent may inhibit proliferation or may be toxic to cells. The term includes agents whose toxic effects are mediated only when transported into the cell and also those whose toxic effect is mediated at the cell surface. A variety of cytotoxic agents can be used and include those that inhibit protein synthesis and those that inhibit expression of certain genes essential for cellular growth or survival. Cytotoxic agents include those that result in cell death and those that inhibit cell growth, proliferation and/or differentiation.

As used herein, ligand refers to any polypeptide that is capable of binding to a cell-surface protein and is capable of facilitating the internalization of a ligand-containing fusion protein into the cell. Such ligands include growth factors, antibodies or fragments thereof, hormones, and other types of proteins.

As used herein, the term "polypeptide reactive with an FGF receptor" refers to any polypeptide that specifically interacts with an FGF receptor, preferably the high-affinity FGF receptor, and that is transported into the cell by virtue of its interaction with the FGF receptor. Polypeptides reactive with an FGF receptor are also referred to herein as FGF proteins. FGF proteins include members of the FGF family of peptides, including FGF-1 through FGF-9, chimeras or hybrids of any of FGF-1 through FGF-9, or FGFs that have deletions (see, e.g., Published International Application No. WO 90/02800, national stage applications, and patents based thereon) or insertions of amino acids, as long as the resulting peptide or protein specifically interacts with an FGF receptor and is internalized by virtue of this interaction.

As used herein, FGF refers to polypeptides having amino acid sequences of native FGF proteins, as well as modified sequences, having amino acid substitutions, deletions, insertions or additions in the native protein but retaining the ability to bind to FGF receptors and to be internalized. Such polypeptides include, but are not limited to, FGF-1 -

FGF-9. For example, bFGF should be generally understood to refer to polypeptides having substantially the same amino acid sequences and receptor-targeting activity as that of bovine bFGF or human bFGF or an acidic FGF. It is understood that differences in amino acid sequences can occur among FGFs of different species as well as among FGFs from individual organisms or species and that not all FGFs bind to all FGF receptor subtypes. It is only required that the FGF bind to at least one FGF receptor.

Reference to FGFs is also intended to encompass proteins isolated from natural sources as well as those made synthetically, as by recombinant means or possibly by chemical synthesis. FGF also encompasses muteins of FGF that possess the ability to target saporin to FGF-receptor expressing cells. Such muteins include, but are not limited to, those produced by replacing one or more of the cysteines with serine as herein or that have any other amino acids deleted or replaced as long as the resulting protein has the ability to bind to FGF-receptor bearing cells and internalize the linked cytotoxic agent. Typically, such muteins will have conservative amino acid changes, such as those set forth below in Table 1. DNA encoding such muteins will, unless modified by replacement of degenerate codons, hybridize under conditions of at least low stringency to DNA encoding bFGF (SEQ ID NO. 12 and 13) or DNA encoding any of the FGF's set forth in SEQ ID. NOs. 24-32.

As used herein, DNA encoding an FGF peptide or polypeptide reactive with an FGF receptor refers to any of the DNA fragments set forth herein as coding such peptides, to any such DNA fragments known to those of skill in the art, any DNA fragment that encodes an FGF that binds to an FGF receptor and is internalized thereby and may be isolated from a human cell library using any of the preceding DNA fragments as a probe any DNA fragment that encodes any of the FGF peptides set forth in SEQ ID NOs. 24-32 (such DNA sequences are available in publicly accessible databases, such as DNA* (July, 1993 release from DNASTAR, Inc.

Madison, WI; see, also U.S. Patent No. 4,956,455, U.S. Patent No. 5,126,323, U.S. Patent No. 5,155,217, U.S. Patent No. 4,868.113, published International Application WO/90/08771 (and the corresponding U.S. patent, upon its issuance), which is based on U.S. Application Serial No. 07/304,281, filed January 31, 1989, and Miyamoto et al. (1993) Mol. Cell. Biol. 13:4251-4259), and any DNA fragment that may be produced from any of the preceding DNA fragments by substitution of degenerate codons. It is understood that once the complete amino acid sequence of a peptide, such as an FGF peptide, and one DNA fragment encoding such peptide are available to those of skill in this art, it is routine to substitute degenerate codons and produce any of the possible DNA fragments that encode such peptide. It is also generally possible to synthesize DNA encoding such peptide based on the amino acid sequence.

As used herein, FGF receptors refer to receptors that specifically interact with a member of the FGF family of proteins and transport it into the cell. Included among these are the receptors described in International Application No. WO 91/00916, which is based on U.S. Patent Application Serial No.07/377,033; International Application No. WO 92/00999, which is based on U.S. Patent Application Serial No.07/549,587; International Application No. WO 90/05522; and International Application No. WO 92/12948; see, also Imamura (1988) Biochem. Biophys. Res. Comm. 155:583-590 and Moscatelli (1987) J. Cell. Physiol. 131:123-130.

As used herein, to target a cytotoxic agent means to direct it to a cell that expresses a selected receptor by linking the agent to a polypeptide reactive with an FGF receptor. Upon binding to the receptor the saporin-containing protein is internalized by the cell and is cytotoxic to the cell.

As used herein, preparations of monogenous conjugates are preparations of conjugates in which each conjugate has the same, generally about 1:1, though not necessarily, molar ratio of targeting molecule to targeted agent. Monogenous conjugates are substantially identical in that they possess indistinguishable chemical and physical properties and

generally preparations of such conjugates contain only one species of conjugate. It is, of course understood, that some variability among the species may be present and will be tolerated to the extent that the activity of each member of the conjugate is substantially the same. For example, saporin that is expressed in bacterial hosts as provided herein may contain a mixture of species that differ at their N-terminus. Such recombinantly produced saporin, however, is suitable for use to produce chemically conjugated conjugates by the methods herein. The resulting preparation is monogenous as defined herein in that each conjugate contains the same molar ratio of FGF protein to targeted agent, but each conjugate is not necessarily identical, but is substantially identical in that each conjugate has substantially the same biological activity.

As used herein, a homogeneous population or composition of conjugates means that the constituent members of the population or composition are monogenous and further do not form aggregates.

As used herein, secretion signal refers to a peptide region within the precursor protein that directs secretion of the precursor protein from the cytoplasm of the host into the periplasmic space or into the extracellular growth medium. Such signals may be either at the amino terminus or carboxyl terminus of the precursor protein. The preferred secretion signal is linked to the amino terminus of the N-terminal extension region.

As used herein, vector or plasmid refers to discrete elements that are used to introduce heterologous DNA into cells for either expression of the heterologous DNA or for replication of the cloned heterologous DNA. Selection and use of such vectors and plasmids are well within the level of skill of the art.

As used herein, expression vector includes vectors capable of expressing DNA fragments that are in operative linkage with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage,

recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or may integrate into the host cell genome.

As used herein, operative linkage or operative association of heterologous DNA to regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences, refers to the functional relationship between such DNA and such sequences of nucleotides. For example, operative linkage of heterologous DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA in reading frame.

As used herein, a promoter region refers to the portion of DNA of a gene that controls transcription of DNA to which it is operatively linked. A portion of the promoter region includes specific sequences of DNA that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of the RNA polymerase. These sequences may be cis acting or may be responsive to trans acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated. For use herein, inducible promoters are preferred. The promoters are recognized by an RNA polymerase that is expressed by the host. The RNA polymerase may be endogenous to the host or may be introduced by genetic engineering into the host, either as part of the host chromosome or on an episomal element, including a plasmid containing the DNA encoding the saporin-containing polypeptide. Most preferred promoters for use herein are tightly

regulated such that, absent induction, the DNA encoding the saporin-containing protein is not expressed.

As used herein, a transcription terminator region has either (a) a subsegment that encodes a polyadenylation signal and polyadenylation site in the transcript, and/or (b) a subsegment that provides a transcription termination signal that terminates transcription by the polymerase that recognizes the selected promoter. The entire transcription terminator may be obtained from a protein-encoding gene, which may be the same or different from the gene, which is the source of the promoter. Preferred transcription terminator regions are those that are functional in E. coli. Transcription terminators are optional components of the expression systems herein, but are employed in preferred embodiments.

As used herein, transfection refers to the taking up of DNA or RNA by a host cell. Transformation refers to this process performed in a manner such that the DNA is replicable, either as an extrachromosomal element or as part of the chromosomal DNA of the host. Methods and means for effecting transfection and transformation are well known to those of skill in this art (see, e.g., Wigler et al. (1979) Proc. Natl. Acad. Sci. USA 76:1373-1376; Cohen et al. (1972) Proc. Natl. Acad. Sci. USA 69:2110).

As used herein, the term biologically active, or reference to the biological activity of a saporin-containing polypeptide or cytotoxicity of a saporin-containing polypeptide, refers to the ability of such polypeptide to inhibit protein synthesis by inactivation of ribosomes either in vivo or in vitro or to inhibit the growth of or kill cells upon internalization of the saporin-containing polypeptide by the cells. Preferred biologically active saporin polypeptides are those that are toxic to eukaryotic cells upon entering the cells. Such biological or cytotoxic activity may be assayed by any method known to those of skill in the art including, but not limited to, the in vitro assays that measure protein synthesis and in vivo assays that assess cytotoxicity by measuring the effect of a test compound on cell

proliferation or on protein synthesis. Particularly preferred, however, are assays that assess cytotoxicity in targeted cells.

As used herein, FGF-mediated pathophysiological condition refers to a deleterious condition characterized by or caused by proliferation of cells that are sensitive to bFGF mitogenic stimulation. Basic FGF-mediated pathophysiological conditions include, but are not limited to, certain tumors, rheumatoid arthritis, restenosis, Dupuytren's Contracture and certain complications of diabetes, such as proliferative retinopathy.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis, high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, isolated, substantially pure DNA refers to DNA fragments purified according to standard techniques employed by those skilled in the art (see, e.g., Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY and Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.).

As used herein, to hybridize under conditions of a specified stringency is used to describe the stability of hybrids formed between two single-stranded DNA fragments and refers to the conditions of ionic strength and temperature at which such hybrids are washed, following annealing under conditions of stringency less than or equal to that of the

washing step. Typically high, medium and low stringency encompass the following conditions or equivalent conditions thereto:

1) high stringency: 0.1 x SSPE or SSC, 0.1% SDS, 65°C

2) medium stringency: 0.2 x SSPE or SSC, 0.1% SDS, 50°C

3) low stringency: 1.0 x SSPE or SSC, 0.1% SDS, 50°C.

Equivalent conditions refer to conditions that select for substantially the same percentage of mismatch in the resulting hybrids. Additions of ingredients, such as formamide, Ficoll, and Denhardt's solution affect parameters such as the temperature under which the hybridization should be conducted and the rate of the reaction. Thus, hybridization in 5 X SSC, in 20% formamide at 42° C is substantially the same as the conditions recited above hybridization under conditions of low stringency. The recipes for SSPE, SSC and Denhardt's and the preparation of deionized formamide are described, for example, in Sambrook et al. (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 8; see, Sambrook et al., vol. 3, p. B.13, see, also, numerous catalogs that describe commonly used laboratory solutions). SSPE is pH 7.4 phosphate-buffered 0.18 NaCl.

As used herein, expression refers to the process by which nucleic acid is transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the nucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

As used herein, "culture" means a propagation of cells in a medium conducive to their growth, and all sub-cultures thereof. The term "subculture" refers to a culture of cells grown from cells of another culture (source culture), or any subculture of the source culture, regardless of the number of subculturings that have been performed between the subculture of interest and the source culture.

As used herein, reference to nucleic acids includes duplex DNA, single-stranded DNA, RNA in any form, including triplex, duplex or single-

stranded RNA, anti-sense RNA, polynucleotides, oligonucleotides, single nucleotides and derivatives thereof.

As used herein an effective amount of a compound for treating a particular disease is an amount that is sufficient to ameliorate, or in some manner reduce the symptoms associated with the disease. Such amount may be administered as a single dosage or may be administered according to a regimen, whereby it is effective. The amount may cure the disease but, typically, is administered in order to ameliorate the symptoms of the disease. Repeated administration may be required to achieve the desired amelioration of symptoms.

As used herein, pharmaceutically acceptable salts, esters or other derivatives of the conjugates include any salts, esters or derivatives that may be readily prepared by those of skill in this art using known methods for such derivatization and that produce compounds that may be administered to animals or humans without substantial toxic effects and that either are pharmaceutically active or are prodrugs.

As used herein, a prodrug is a compound that, upon in vivo administration, is metabolized or otherwise converted to the biologically, pharmaceutically or therapeutically active form of the compound. To produce a prodrug, the pharmaceutically active compound is modified such that the active compound will be regenerated by metabolic processes. The prodrug may be designed to alter the metabolic stability or the transport characteristics of a drug, to mask side effects or toxicity, to improve the flavor of a drug or to alter other characteristics or properties of a drug. By virtue of knowledge of pharmacodynamic processes and drug metabolism in vivo, those of skill in this art, once a pharmaceutically active compound is known, can design prodrugs of the compound (see, e.g., Nogrady (1985) Medicinal Chemistry A Biochemical Approach, Oxford University Press, New York, pages 388-392).

As used herein, treatment means any manner in which the symptoms of a condition, disorder or disease are ameliorated or otherwise

beneficially altered. Treatment also encompasses any pharmaceutical use of the compositions herein.

As used herein, amelioration of the symptoms of a particular disorder by administration of a particular pharmaceutical composition refers to any lessening, whether permanent or temporary, lasting or transient that can be attributed to or associated with administration of the composition.

As used herein, ED_{50} refers to the concentration at which 50% of the cells are killed following incubation, generally for 72-hours or other specified time period, with a toxin, such as FGF-SAP.

As used herein, ID_{50} refers to the concentration of saporin-containing protein required to inhibit protein synthesis in treated cells to 50% of the protein synthesis in the absence of the protein.

A. Preparation of polypeptides and cytotoxic agents

1. Polypeptides reactive with an FGF receptor

Any polypeptide that is reactive with an FGF receptor may be used in the methods herein. Members of the FGF peptide family, including FGF-1 - FGF-9, are particularly preferred. Modification of the polypeptide may be effected by any means known to those of skill in this art. The preferred methods herein rely on modification of DNA encoding the polypeptide and expression of the modified DNA.

DNA encoding the FGF polypeptide may be isolated, synthesized or obtained from commercial sources (the amino acid sequences of FGF-1 - FGF-9 are set forth in SEQ ID NOs. 24-32; DNA sequences may be based on these amino acid sequences or may be those that are known to those of skill in this art (see, e.g., DNA* (July, 1993 release from DNASTAR, Inc. Madison, WI); see, also U.S. Patent No. 4,956,455, U.S. Patent No. 5,126,323, U.S. Patent No. 5,155,217, U.S. Patent No. 4,868.113, published International Application WO/90/08771 (and the corresponding U.S. patent, upon its issuance), which is based on U.S. Application Serial No. 07/304,281, filed January 31, 1989, and Miyamoto et al. (1993) Mol. Cell. Biol. 13:4251-4259)) Expression of a recombinant bFGF protein in

yeast and E. coli is described in Barr et al., J. Biol. Chem. 263:16471-16478 (1988), in copending International PCT Application Serial No. PCT/US93/05702 and co-pending United States Application Serial No. 07/901,718. Expression of recombinant FGF proteins may be performed as described herein; and DNA encoding FGF proteins may be used as the starting materials for the methods herein.

Mutation may be effected by any method known to those of skill in the art, including site-specific or site-directed mutagenesis of DNA encoding the protein and the use of DNA amplification methods using primers to introduce and amplify alterations in the DNA template. Site-specific mutagenesis is typically effected using a phage vector that has single- and double-stranded forms, such as M13 phage vectors, which are well-known and commercially available. Other suitable vectors that contain a single-stranded phage origin of replication may be used (see, e.g., Veira et al. (1987) Meth. Enzymol. 15:3). In general, site-directed mutagenesis is performed by preparing a single-stranded vector that encodes the protein of interest (i.e., a member of the FGF family or a cytotoxic molecule, such as a saporin). An oligonucleotide primer that contains the desired mutation within a region of homology to the DNA in the single-stranded vector is annealed to the vector followed by addition of a DNA polymerase, such as E. coli polymerase I Klenow fragment, which uses the double stranded region as a primer to produce a heteroduplex in which one strand encodes the altered sequence and the other the original sequence. The heteroduplex is introduced into appropriate bacterial cells and clones that include the desired mutation are selected. The resulting altered DNA molecules may be expressed recombinantly in appropriate host cells to produce the modified protein.

Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential

regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. *Molecular Biology of the Gene*, 4th Edition, 1987, The Bejacmin/Cummings Pub. co., p.224).

Such substitutions are preferably made in accordance with those set forth in TABLE 1 as follows:

TABLE 1

Original residue	Conservative substitution
Ala (A)	Gly; Ser
Arg (R)	Lys
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions.

2. The cytotoxic agent

Saporin and other ribosome inactivating proteins (RIPs) are the preferred cytotoxic agent for use herein. Any cytotoxic agent that, when internalized inhibits or destroys cell growth, cell proliferation or other essential cell functions may be used herein. Such cytotoxic agents are considered to be functionally equivalent to the RIPs described herein, and include, but are not limited to, saporin, the ricins, abrin and other RIPs, Pseudomonas exotoxin, inhibitors of DNA, RNA or protein synthesis or other metabolic inhibitors that are known to those of skill in this art. Saporin is preferred, but other suitable RIPs include, but are not limited to, ricin, ricin A chain, maize RIP, gelonin, diphtheria toxin, diphtheria toxin A

chain, trichosanthin, tritin, pokeweed antiviral protein (PAP), mirabilis antiviral protein (MAP), Dianthins 32 and 30, abrin, monordin, bryodin, shiga and others known to those of skill in this art (see, e.g., Barbieri et al. (1982) Cancer Surveys 1:489-520 and European published patent application No. 0466 222, incorporated herein by reference, which provide lists of numerous RIPs and their sources; see, also, U.S. Patent No. 5,248,608 to Walsh et al., which provides a RIP from maize).

The selected cytotoxic agent is, if necessary, derivatized to produce a group reactive with a cysteine on the selected FGF. If derivatization results in a mixture of reactive species, a mono-derivatized form of the cytotoxic agent is isolated and is then conjugated to the mutated FGF.

a. Isolation of saporin and DNA encoding saporin

Saporin is preferred herein. The saporin polypeptides include any of the isoforms of saporin that may be isolated from Saponaria officinalis or related species or modified form that retain cytotoxic activity. In particular, such modified saporin may be produced by modifying the DNA encoding the protein (see, e.g., International PCT Application Serial No. PCT/US93/05702, filed on June 14, 1993, which is a continuation-in-part of United States Application Serial No. 07/901,718; see, also, copending U.S. Patent Application No. 07/885,242 filed May 20, 1992, and Patent No. 1231914, granted in Italy on January 15, 1992) by altering one or more amino acids or deleting or inserting one or more amino acids, such as a cysteine that may render it easier to conjugate to FGF or other cell surface binding protein. Any such protein, or portion thereof, that, when conjugated to FGF as described herein, that exhibits cytotoxicity in standard in vitro or in vivo assays within at least about an order of magnitude of the saporin conjugates described herein is contemplated for use herein.

Thus, the SAP used herein includes any protein that is isolated from natural sources or that is produced by recombinant expression (see, e.g., copending International PCT Application Serial No. PCT/US93/05702, filed

on June 14, 1993, which is a continuation-in-part of United States Application Serial No. 07/901,718, filed June 16, 1992; see, also Example 1, below).

DNA encoding SAP or any cytotoxic agent may be used in the recombinant methods provided herein. In instances in which the cytotoxic agent does not contain a cysteine residue, such as instances in which DNA encoding SAP is selected, the DNA may be modified to include cysteine codon. The codon may be inserted into any locus that does not reduce or reduces by less than about one order of magnitude the cytotoxicity of the resulting protein may be selected. Such locus may be determined empirically by modifying the protein and testing it for cytotoxicity in an assay, such as a cell-free protein synthesis assay. The preferred loci in SAP for insertion of the cysteine residue is at or near the N-terminus (within about 10 residues of the N-terminus).

b. Host cells for expression of saporin containing polypeptides

Host organisms include those organisms in which recombinant production of heterologous proteins have been carried out, such as, but not limited to, bacteria (for example, E. coli), yeast (for example, Saccharomyces cerevisiae and Pichia pastoris), mammalian cells, insect cells. Presently preferred host organisms are strains of bacteria. Most preferred host organisms are strains of E. coli.

c. Methods for recombinant production of saporin

The DNA encoding the cytotoxic agent, such as saporin protein, is introduced into a plasmid in operative linkage to an appropriate promoter for expression of polypeptides in a selected host organism. The presently preferred saporin proteins are saporin proteins that have been modified by addition of a Cys residue or replacement of a non-essential residue at or near the amino- or carboxyl terminus of the saporin with Cys. Saporin, such as that of SEQ ID NO. 7 has been modified by insertion of Met-Cys residue at the N-terminus and has also been modified by replacement of the

Asn or Ile residue at positions 4 and 10, respectively (see EXAMPLE 4). The DNA fragment encoding the saporin may also include a protein secretion signal that functions in the selected host to direct the mature polypeptide into the periplasm or culture medium. The resulting saporin protein can be purified by methods routinely used in the art, including, methods described hereinafter in the Examples.

Methods of transforming suitable host cells, preferably bacterial cells, and more preferably E. coli cells, as well as methods applicable for culturing said cells containing a gene encoding a heterologous protein, are generally known in the art. See, for example, Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

The DNA construct encoding the saporin protein is introduced into the host cell by any suitable means, including, but not limited to transformation employing plasmids, viral, or bacterial phage vectors, transfection, electroporation, lipofection, and the like. The heterologous DNA can optionally include sequences, such as origins of replication that allow for the extrachromosomal maintenance of the saporin-containing plasmid, or can be designed to integrate into the genome of the host (as an alternative means to ensure stable maintenance in the host).

Positive transformants can be characterized by Southern blot analysis (Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) for the site of DNA integration; Northern blots for inducible-promoter-responsive saporin gene expression; and product analysis for the presence of saporin-containing proteins in either the cytoplasm, periplasm, or the growth media.

Once the saporin-encoding DNA fragment has been introduced into the host cell, the desired saporin-containing protein is produced by subjecting the host cell to conditions under which the promoter is induced, whereby the operatively linked DNA is transcribed. In a preferred embodiment, such conditions are those that induce expression from the E.

coli lac operon. The plasmid containing the DNA encoding the saporin-containing protein also includes the lac operator (O) region within the promoter and may also include the lac I gene encoding the lac repressor protein (see, e.g., Muller-Hill et al. (1968) Proc. Natl. Acad. Sci. USA 59:1259-12649). The lac repressor represses the expression from the lac promoter until induced by the addition of IPTG in an amount sufficient to induce transcription of the DNA encoding the saporin-containing protein.

The expression of saporin in E. coli is, thus accomplished in a two-stage process. In the first stage, a culture of transformed E. coli cells is grown under conditions in which the expression of the saporin-containing protein within the transforming plasmid, preferably a encoding a saporin, such as described in Example 4, is repressed by virtue of the lac repressor. In this stage cell density increases. When an optimum density is reached, the second stage commences by addition of IPTG, which prevents binding of repressor to the operator thereby inducing the lac promoter and transcription of the saporin-encoding DNA.

In a preferred embodiment, the promoter is the T7 RNA polymerase promoter, which may be linked to the lac operator and the E. coli host strain includes DNA encoding T7 RNA polymerase operably linked to the lac operator and a promoter, preferably the lacUV5 promoter. The presently preferred plasmid is pET 11a (NOVAGEN, Madison, WI), which contains the T7lac promoter, T7 terminator, the inducible E. coli lac operator, and the lac repressor gene. The plasmid pET 15b (NOVAGEN, Madison, WI), which contains a His-Tag™ leader sequence (Seq. ID No. 36) for use in purification with a His column and a thrombin cleavage site that permits cleavage following purification over the column, the T7-lac promoter region and the T7 terminator, has been used herein for expression of saporin. Addition of IPTG induces expression of the T7 RNA polymerase and the T7 promoter, which is recognized by the T7 RNA polymerase.

Transformed strains, which are of the desired phenotype and genotype, are grown in fermentors by suitable methods well known in the

art. In the first, or growth stage, expression hosts are cultured in defined minimal medium lacking the inducing condition, preferably IPTG. When grown in such conditions, heterologous gene expression is completely repressed, which allows the generation of cell mass in the absence of heterologous protein expression. Subsequent to the period of growth under repression of heterologous gene expression, the inducer, preferably IPTG, is added to the fermentation broth, thereby inducing expression of any DNA operatively linked to an IPTG-responsive promoter (a promoter region that contains lac operator). This last stage is the induction stage.

The resulting saporin-containing protein can be suitably isolated from the other fermentation products by methods routinely used in the art, e.g., using a suitable affinity column as described in Example 1.E-F and 2.D; precipitation with ammonium sulfate; gel filtration; chromatography, preparative flat-bed iso-electric focusing; gel electrophoresis, high performance liquid chromatography (HPLC); and the like. A method for isolating saporin is provided in EXAMPLE 1 (see, also Lappi et al. (1985) Biochem. Biophys. Res. Commun. 129:934-942). The expressed saporin protein is isolated from either the cytoplasm, periplasm, or the cell culture medium (see, discussion below B.1.b below and see, e.g., EXAMPLE 4 for preferred methods and saporin proteins).

3. Plasmids for expression of the FGF peptide, the cytotoxic agent and FGF peptide-cytotoxic agent chimeras

The DNA construct is introduced into a plasmid for expression in a desired host. In preferred embodiments, the host is a bacterial host. The sequences of nucleotides in the plasmids that are regulatory regions, such as promoters and operators, are operationally associated with one another for transcription of the sequence of nucleotides that encode a saporin-containing protein. The sequence of nucleotides encoding the saporin-containing protein may also include DNA encoding a secretion signal, whereby the resulting peptide is a precursor of saporin. The resulting processed saporin protein, which if not processed such that the

resulting protein is identical to a native saporin, retains the cytotoxic activity of the native saporin protein, may be recovered from the periplasmic space or the fermentation medium.

In preferred embodiments the DNA plasmids also include a transcription terminator sequence. The promoter regions and transcription terminators are each independently selected from the same or different genes.

The plasmids used herein preferably include a promoter in operable association with the DNA encoding the saporin-containing protein and are designed for expression of proteins in a bacterial host. It has been found that tightly regulatable promoters are preferred for expression of saporin. Suitable promoters for expression of saporin-containing proteins are widely available and are well known in the art. Inducible promoters or constitutive promoters that are linked to regulatory regions are preferred. Such promoters include, but are not limited to, the T7 phage promoter and other T7-like phage promoters, such as the T3, T5 and SP6 promoters, the trp, lpp, and lac promoters, such as the lacUV5, from E. coli; the P10 or polyhedron gene promoter of baculovirus/insect cell expression systems and inducible promoters from other eukaryotic expression systems. For expression of the saporin-containing proteins such promoters are inserted in a plasmid in operative linkage with a control region such as the lac operon.

Preferred promoter regions are those that are inducible and functional in E. coli. Examples of suitable inducible promoters and promoter regions include, but are not limited to: the E. coli lac operator responsive to isopropyl β-D-thiogalactopyranoside (IPTG; see, et al. Nakamura et al. (1979) Cell 18:1109-1117); the metallothionein promoter metal-regulatory-elements responsive to heavy-metal (e.g., zinc) induction (see, e.g., U.S. Patent No. 4,870,009 to Evans et al.); and the phage T7lac promoter responsive to IPTG (see, e.g., U.S. Patent No. 4,952,496; and Studier _t al. (1990) Meth. Enzymol. 185:60-89).

The plasmids also preferably include a selectable marker gene or genes that are functional in the host. A selectable marker gene includes any gene that confers a phenotype on bacteria that allows transformed bacterial cells to be identified and selectively grown from among a vast majority of untransformed cells. Suitable selectable marker genes for bacterial hosts, for example, include the ampicillin resistance gene (Amp^r), tetracycline resistance gene (Tc^r) and the kanamycin resistance gene (Kan^r). The kanamycin resistance gene is presently preferred.

The preferred plasmids also include DNA encoding a signal for secretion of the operably saporin-containing protein. Secretion signals suitable for use are widely available and are well known in the art. Prokaryotic and eukaryotic secretion signals functional in E. coli may be employed. The presently preferred secretion signals include, but are not limited to, those encoded by the following E. coli genes: ompA, ompT, ompF, ompC, beta-lactamase, and alkaline phosphatase, and the like (von Heijne (1985) J. Mol. Biol. 184:99-105). In addition, the bacterial pelB gene secretion signal (Lei et al. (1987) J. Bacteriol. 169:4379), the phoA secretion signal, and the cek2 functional in insect cell may be employed. The most preferred secretion signal is the E. coli ompA secretion signal. Other prokaryotic and eukaryotic secretion signals known to those of skill in the art may also be employed (see, e.g., von Heijne (1985) J. Mol. Biol. 184:99-105). Using the methods described herein, one of skill in the art can substitute secretion signals that are functional in either yeast, insect or mammalian cells to secrete saporin-containing proteins from those cells.

Particularly preferred plasmids for transformation of E. coli cells include the pET expression vectors (see, U.S patent 4,952,496; available from NOVAGEN, Madison, WI). Such plasmids include pET 11a, which contains the T7lac promoter, T7 terminator, the inducible E. coli lac operator, and the lac repressor gene; pET 12a-c, which contains the T7 promoter, T7 terminator, and the E. coli ompT secretion signal; and pET 15b (NOVAGEN, Madison, WI), which contains a His-Tag™ leader

sequence (Seq. ID No. 36) for use in purification with a His column and a thrombin cleavage site that permits cleavage following purification over the column; the T7-lac promoter region and the T7 terminator.

Other preferred plasmids include the pKK plasmids, particularly pKK 223-3, which contains the TAC promoter, (available from Pharmacia; see also, Brosius et al. (1984) Proc.. Natl. Acad. Sci. 81:6929; Ausubel et al., Current Protocols in Molecular Biology; U.S. Patent Nos. 5,122,463, 5,173,403, 5,187,153, 5,204,254, 5,212,058, 5,212,286, 5,215,907, 5,220,013, 5,223,483, and 5,229,279), which contain the TAC promoter. Plasmid pKK has been modified by disruption of the ampicillin resistance marker gene by digestion with ScaI and insertion of a kanamycin resistance cassette (purchased from Pharmacia; obtained from pUC4K, see, e.g., Vieira et al. (1982) Gene 19:259-268; and U.S. Patent No. 4,719,179) cut with HincII to remove the EcoRI sticky ends and produce blunt ends. Baculovirus vectors, such as a pBlueBac (also called pJVETL and derivatives thereof) vector, particularly pBlueBac III, (see, e.g., U.S. Patent Nos. 5,278,050, 5,244,805, 5,243,041, 5,242,687, 5,266,317, 4,745,051, and 5,169,784; available from INVITROGEN, San Diego) may also be used for expression of the polypeptides in insect cells. The pBlueBacIII vector is a dual promoter vector and provides for the selection of recombinants by blue/white screening as this plasmid contains the β-galactosidase gene (lacZ) under the control of the insect recognizable ETL promoter and is inducible with IPTG. A DNA construct is inserted into a baculovirus vector pBluebac III (INVITROGEN, San Diego, CA) and then co-transfected with wild type virus into insect cells Spodoptera frugiperda (sf9 cells; see, e.g., Luckow et al. (1988) Bio/technology 6:47-55 and U.S. Patent No. 4,745,051).

Other plasmids include the pIN-IIIompA plasmids (see, U.S. Patent No. 4,575,013 to Inouye; see, also, Duffaud et al. (1987) Meth. Enz. 153:492-507), such as pIN-IIIompA2 . The pIN-IIIompA plasmids include an insertion site for the heterologous DNA (the DNA encoding a saporin-

containing protein) linked for transcriptional expression in reading phase with four functional fragments derived from the lipoprotein gene of E. coli. The plasmids also include a DNA fragment coding for the signal peptide of the ompA protein of E. coli, positioned such that the desired polypeptide is expressed with the ompA signal peptide at its amino terminus, thereby allowing efficient secretion across the cytoplasmic membrane. The plasmids further include DNA encoding a specific segment of the E. coli lac promoter-operator, which is positioned in the proper orientation for transcriptional expression of the desired polypeptide, as well as a separate functional E. coli lacI gene encoding the associated repressor molecule that, in the absence of lac operon inducer, interacts with the lac promoter-operator to prevent transcription therefrom. Expression of the desired polypeptide is under the control of the lipoprotein (lpp) promoter and the lac promoter-operator, although transcription from either promoter is normally blocked by the repressor molecule. The repressor is selectively inactivated by means of an inducer molecule thereby inducing transcriptional expression of the desired polypeptide from both promoters.

In a preferred embodiment, the DNA fragment is replicated in bacterial cells, preferably in E. coli. The preferred DNA fragment also includes a bacterial origin of replication, to ensure the maintenance of the DNA fragment from generation to generation of the bacteria. In this way, large quantities of the DNA fragment can be produced by replication in bacteria. Preferred bacterial origins of replication include, but are not limited to, the f1-ori and col E1 origins of replication. Preferred hosts contain chromosomal copies of DNA encoding T7 RNA polymerase operably linked to an inducible promoter, such as the lacUV promoter (see, U.S. Patent No. 4,952,496). Such hosts include, but are not limited to, lysogens E. coli strains HMS174(DE3)pLysS, BL21(DE3)pLysS, HMS174(DE3) and BL21(DE3). Strain BL21(DE3) is preferred. The pLys strains provide low levels of T7 lysozyme, a natural inhibitor of T7 RNA polymerase.

The DNA fragments provided may also contain a gene coding for a repressor-protein. The repressor-protein is capable of repressing the transcription of a promoter that contains sequences of nucleotides to which the repressor-protein binds. The promoter can be derepressed by altering the physiological conditions of the cell. The alteration can be accomplished by the addition to the growth medium of a molecule that inhibits, for example, the ability to interact with the operator or with regulatory proteins or other regions of the DNA or by altering the temperature of the growth media. Preferred repressor-proteins include, but are not limited to, the E. coli. lacI repressor responsive to IPTG induction, the temperature sensitive cI857 repressor, and the like. The E. coli lacI repressor is preferred.

DNA encoding full-length bFGF or the bFGF muteins has been linked to DNA encoding the mature saporin protein and introduced into the pET vectors, including pET-11a and pET-12a expression vectors (NOVAGEN, Madison, WI), for intracellular and periplasmic expression, respectively, of FGF-SAP fusion proteins. The resulting fusion proteins exhibit cytotoxic activity and appear to be at least as potent as the chemically conjugated FGF-SAP preparations. The resulting bFGF-fusion proteins are highly cytotoxic when internalized by targeted cells.

B. Synthesis of monogenous preparations of cytotoxic conjugates and homogeneous populations of cytotoxic conjugates

The problem of heterogeneity of compositions and preparations of cytotoxic FGF conjugates has been addressed in several ways herein. The first method relies on chemical conjugation and the second method relies on recombinant DNA technology. The methods herein are described with respect to bFGF and SAP. It is understood, however, that the same methods may be used to modify and prepare homogeneous populations of conjugates of any member of the FGF family with SAP, modified SAP, or any other cytotoxic agent.

1. **Chemical conjugation**

To effect chemical conjugation herein, the FGF protein is modified and then linked to the cytotoxic agent. Chemical conjugation must be used if the cytotoxic agent is other than a peptide or protein, such as a non-peptide drug.

a. Selection of the FGF protein

To reduce the heterogeneity of preparations of FGF protein-containing chemical conjugates, the FGF protein is modified by deleting or replacing a site(s) on the FGF that causes the heterogeneity. Such sites are typically cysteine residues that, upon folding of the protein, remain available for interaction with other cysteines or for interaction with more than one cytotoxic molecule per molecule of FGF peptide. Thus, such cysteine residues do not include any cysteine residue that are required for proper folding of the FGF peptide or for retention of the ability to bind to an FGF receptor and internalize. For chemical conjugation, one cysteine residue that, in physiological conditions, is available for interaction, is not replaced because it is used as the site for linking the cytotoxic moiety. The resulting modified FGF is conjugated with a single species of cytotoxic conjugate.

Any protein that is reactive with an FGF receptor may be used herein. In particular any of FGF-1 - FGF-9 may be modified for use herein or reacted with a cytotoxic reagent, such that the resulting conjugate is monogenous. FGF-1 has cysteines at positions 31, 98 and 132; FGF-2 has cysteines at positions 34, 78, 96 and 101; FGF-3 has cysteines at positions 50 and 115; FGF-4 has cysteines at positions 88 and 155; FGF-5 has cysteines at positions 19, 93, 160 and 202; FGF-6 has cysteines at positions 80 and 147; FGF-7 has cysteines at positions 18, 23, 32, 46, 71, 133 and 137; FGF-8 has cysteines at positions 10, 19, 109 and 127; and FGF-9 has cysteines at positions 68 and 134. The cysteine residues from each of FGF-1 - FGF-9 that appear to be essential for retention of biological activity and that should not deleted or replaced are as follows:

TABLE 2

FGF-1	cys^{98}
FGF-2	cys^{101}
FGF-3	cys^{115}
FGF-4	cys^{155}
FGF-5	cys^{160}
FGF-6	cys^{147}
FGF-7	cys^{137}
FGF-8	cys^{127}
FGF-9	cys^{134}.

The FGF peptides may be modified as described below. Alternatively, the contribution of each cysteine to the ability to bind to FGF receptors may be determined empirically. Each cysteine residue may be systematically replaced with a conservative amino acid change (see Table 1, above) or deleted. The resulting mutein is tested for the requisite biological activity, the ability to bind to FGF receptors and internalize linked cytotoxic moieties. If the mutein retains this activity, then the cysteine residue is not required. Additional cysteines are systematically deleted and replaced and the resulting muteins are tested for activity. In this manner the minimum number and identity of the cysteines needed to retain the ability to bind to an FGF receptor and internalize may be determined.

For each FGF peptide, the complete amino acid sequence is known (see, e.g., SEQ ID NO. 24 (FGF-1) and SEQ ID NOs. 26-32 (FGF-3 - FGF-9, respectively)). The sequence is examined and cysteine residues are identified. Comparison among the amino acid sequences of FGF-1 -FGF-9 reveals that one Cys is conserved among FGF family of peptides (see Table 2). These cysteine residues may be required for secondary structure and should be altered. These residues should not be replaced. Each of the remaining cysteine residues may be systematically deleted and/or replaced by a serine residue or other residue that would not be expected to alter the structure of the protein. The resulting peptide is tested for biological activity. If the cysteine residue is necessary for retention of biological activity it is not deleted; if it is not necessary, then it is preferably replaced

with a serine or other residue selected so that it does not alter the secondary structure of the resulting protein.

b. Modification of the FGF protein for chemical conjugation

The polypeptide reactive with an FGF receptor is modified by removing one or more reactive cysteines that are not required for receptor binding, but that are available for reaction with appropriately derivatized cytotoxic agent, so that the resulting FGF protein has only one cysteine residue available for conjugation with the cytotoxic agent. Other cysteine residues are removed and, preferably, replaced with an amino acid that does not substantially alter the biological activity of the resulting mutant FGF. The resulting mutant FGF is then tested for retention of the ability to target a cytotoxic agent to a cell that expresses an FGF receptor and to internalize the cytotoxic agent into such cells. Retention of proliferative activity is indicative, though not definitive, of the retention of such activities. Proliferative activity may be measured by any suitable proliferation assay, such as the assay, exemplified below, that measures the increase in cell number of adrenal capillary endothelial cells. It is noted, however, that modified or mutant FGFs may exhibit reduced or no proliferative activity, but may be suitable for use herein, if they retain the ability to target a linked cytotoxic agent to cells bearing receptors to which the unmodified FGF binds and result in internalization of the cytotoxic moiety.

Since FGF-3, FGF-4 and FGF-6 have only two cysteines, for purposes of chemical conjugation, preferably neither cysteine is deleted or replaced, unless another residue, preferably one near either terminus, is replaced with a cysteine. With respect to the other FGF family members, at least one cysteine must remain available for conjugation with the cytotoxic conjugate and probably two cysteines, but at least the cysteine residues set forth in Table 2. A second cysteine may be required to form a disulfide bond. Thus, any FGF peptide that has more than three cysteines is be modified for chemical conjugation by deleting or replacing the other

cysteine residues. FGF peptides that have three cysteine residues are modified by elimination of one cysteine, conjugated to a cytotoxic moiety and tested for the ability to bind to FGF receptors and internalize the cytotoxic moiety.

In accord with the methods herein, two muteins of basic FGF for chemical conjugation have been produced (preparation of muteins for recombinant expression of the conjugate is described below). DNA, obtained from pFC80 (see, copending International PCT Application Serial No. PCT/US93/05702, which is a continuation-in-part of United States Application Serial No. 07/901,718; see also, SEQ ID NO. 12) encoding basic FGF has been mutagenized. Mutagenesis of cysteine 78 of basic FGF to serine ([C78S]FGF) or cysteine 96 to serine ([C96S]FGF) produced two mutants that retain virtually complete proliferative activity of native basic FGF as judged by the ability to stimulate endothelial cell proliferation in culture. The activities of the two mutants and the native protein do not significantly differ as assessed by efficacy or maximal response. Sequence analysis of the modified DNA verified that each of the mutants has one codon for cysteine converted to that for serine.

The resulting mutein FGF or unmodified FGF is reacted with a single species of cytotoxic agent. The bFGF muteins have been reacted with a single species of derivatized saporin (mono-derivatized saporin) thereby resulting in monogenous preparations of FGF-SAP conjugates and homogeneous compositions of FGF-SAP chemical conjugates. The resulting chemical conjugate does not aggregate and retains the requisite biological activities.

c. Preparation of saporin

(1) Isolation of mono-derivatized SAP.

For chemical conjugation, the SAP may be derivatized or modified such that it includes a cysteine residue for conjugation to the FGF protein.

Typically, SAP is derivatized by reaction with SPDP. This results in a heterogeneous population. For example, SAP that is derivatized by SPDP

to a level of 0.9 moles pyridine-disulfide per mole of SAP includes a population of non-derivatized, mono-derivatized and di-derivatized SAP. Ribosome-inactivating proteins, which are overly derivatized with SPDP, may lose activity because of reaction with sensitive lysines (Lambert et al. (1988) Cancer Treat. Res. 37:175-209). The quantity of non-derivatized SAP in the preparation of the non-purified material can be difficult to judge and this may lead to errors in being able to estimate the correct proportion of derivatized SAP to add to the reaction mixture.

Because of the removal of a negative charge by the reaction of SPDP with lysine, the three species, however, have a charge difference. The methods herein rely on this charge difference for purification of mono-derivatized SAP by Mono S cation exchange chromatography. The use of purified mono-derivatized SAP has distinct advantages over the non-purified material. The amount of basic FGF that can react with SAP is limited to one molecule with the mono-derivatized material, and it is seen in the results presented herein that a more homogeneous conjugate is produced. There are still sources of heterogeneity with the mono-derivatized SAP used here. Native SAP as purified from the seed itself is a mixture of four isoforms, as judged by protein sequencing (see, e.g., International PCT Application Serial No. PCT/US93/05702 and copending United States Application Serial No. 07/901,718; see also, Montecucchi et al. (1989) Int. J. Pept. Prot. Res. 33:263-267; Maras et al. (1990) Biochem. Internat. 21:631-638; and Barra et al. (1991) Biotechnol. Appl. Biochem. 13:48-53). This creates some heterogeneity in the conjugates, since the reaction with SPDP probably occurs equally with each isoform. This source of heterogeneity can be overcome, for example, by use of SAP expressed in E. coli.

(2) Recombinant expression of saporin

DNA provided herein includes a sequence of nucleotides encoding a saporin polypeptide and an N-terminal extension sequence linked to the amino terminus of the saporin. The N-terminal extension permits

expression of saporin in a bacterial host. If saporin is linked to DNA encoding an FGF peptide, then the N-terminal extension is not necessary, but may be included and contain from about one up to 20-30 amino acid residues or more, if desired, and as long as the resulting saporin peptide retains cytotoxic activity.

Suitable N-terminal extension regions may be substantially neutral and lack any biological function other than rendering the saporin polypeptide nontoxic or less toxic to the host in which it is expressed. The specific amino acid makeup of the N-terminal extension region does not appear to be critical for rendering the saporin-containing protein nontoxic or less toxic to the host upon expression of the protein.

In a preferred embodiment, the N-terminal extension region is susceptible to cleavage by eukaryotic intracellular proteases, either by general intracellular degradation or by site-specific proteolytic processing of a proteolytic signal sequence such that, upon internalization, the N-terminal extension region of the saporin-containing fusion protein is cleaved or degraded by a cellular eukaryotic protease, which renders the single-fragment saporin protein biologically active, resulting in cell death (see, e.g., copending U.S. Application 08/ , , filed concurrently herewith).

The DNA molecules provided herein encode saporin that has substantially the same amino acid sequence and ribosome-inactivating activity as that of saporin-6 (SO-6), including any of four isoforms, which have heterogeneity at amino acid positions 48 and 91 (see, e.g., Maras et al. (1990) Biochem. Internat. 21:631-638 and Barra et al. (1991) Biotechnol. Appl. Biochem. 13:48-53 and SEQ ID NOs. 3-7). Other suitable saporin polypeptides include other members of the multi-gene family coding for isoforms of saporin-type RIP's including SO-1 and SO-3 (Fordham-Skelton et al. (1990) Mol. Gen. Genet. 221:134-138), SO-2 (see, e.g., U.S. Application Serial No. 07/885,242, which corresponds to GB 2,216,891; see, also, Fordham-Skelton et al. (1991) Mol. Gen. Genet.

229:460-466), SO-4 (see, e.g., GB 2,194,241 B; see, also, Lappi et al. (1985) Biochem. Biophys. Res. Commun. 129:934-942) and SO-5 (see, e.g., GB 2,194,241 B; see, also, Montecucchi et al. (1989) Int. J. Peptide Protein Res. 33:263-267). SO-4, which includes the N-terminal 40 amino acids set forth in SEQ ID NO. 33, is isolated from the leaves of Saponaria officinalis by extraction with 0.1 M phosphate buffer at pH 7, followed by dialysis of the supernatant against sodium borate buffer, pH 9, and selective elution from a negatively charged ion exchange resin, such as Mono S (Pharmacia Fine Chemicals, Sweden) using gradient of 1 to 0.3 M. NaCl and first eluting chromatographic fraction that has SAP activity. The second eluting fraction is SO-5.

The saporin polypeptides exemplified herein include those having substantially the same amino acid sequence as those listed in SEQ ID NOs 3-7. The isolation and expression of the DNA encoding these proteins is described in Example 1.

(3) Modification of saporin

Because more than one amino group on SAP may react with the succinimidyl moiety, it is possible that more than one amino group on the surface of the protein is reactive. This creates the potential for heterogeneity in the mono-derivatized SAP. This source of heterogeneity has been solved by the conjugating modified SAP expressed in E. coli that has an additional cysteine inserted, as described above, in the coding sequence.

Thus, in other embodiments, instead of derivatizing saporin to introduce a sulfhydryl, the saporin can be modified by the introduction of a cysteine residue into the SAP such that the resulting modified saporin protein reacts with the FGF protein to produce a monogenous cytotoxic conjugate that binds to FGF receptors on eukaryotic cells and is cytotoxic upon internalization by such cells. Preferred loci for introduction of a cysteine residue include the N-terminus region, preferably within about one to twenty residues from the N-terminus of the cytotoxic agent, such as

SAP. For expression of SAP in the bacterial host systems herein, it is also desirable to add DNA encoding a methionine linked to the DNA encoding the N-terminus of the saporin protein. DNA encoding SAP has been modified by inserting a DNA encoding Met-Cys (ATG TGT or ATG TGC) at the N-terminus immediately adjacent to the codon for first residue of the mature protein.

Muteins in which a cysteine residue has been added at the N-terminus and muteins in which the amino acid at position 4 or 10 has been replaced with cysteine have been prepared by modifying the DNA encoding saporin (see, EXAMPLE 4). The modified DNA may be expressed and the resulting saporin protein purified, as described herein for expression and purification of the resulting SAP. The modified saporin can then be reacted with the modified FGF to form disulfide linkages between the single exposed cysteine residue on the FGF and the cysteine residue on the modified SAP.

The modified DNA may be expressed and the resulting saporin protein purified, as described herein for expression and purification of the resulting SAP. The modified saporin can then be reacted with the modified FGF to form disulfide linkages between the single exposed cysteine residue on the FGF and the cysteine residue on the modified SAP.

Using either methodology (reacting mono-derivatized SAP with the FGF peptide or introducing a cys residue into SAP), the resulting preparations of FGF-SAP chemical conjugates are monogenous; compositions containing the conjugates also appear to be free of aggregates.

The above-described sources for heterogeneity also can be avoided by producing the cytotoxic conjugate as a fusion protein by expression of DNA encoding the modified FGF protein linked to DNA encoding the cytotoxic agent, as described below.

2. Recombinant production of cytotoxic conjugates containing modified FGF

The problem of heterogeneity has also been addressed herein by preparing the conjugates as fusion proteins using recombinant DNA technology. Preparations containing the fusion proteins may be rendered more homogeneous by modifying the FGF and/or the targeted agent to prevent interactions between each conjugate, such as via unreacted cysteines. Expression of DNA encoding a fusion of an FGF protein linked to the cytotoxic agent results in a monogenous preparation of cytotoxic conjugates. Such population may, however, form aggregates. Preparations containing the fusion proteins may be rendered more homogeneous by modifying the FGF and/or the cytotoxic agent to prevent interactions between each conjugate, such as via unreacted cysteines. Aggregate formation has been eliminated by preparing mutein constructs in which the cysteine residues on the FGF are deleted or replaced. Cytotoxic conjugates containing bFGF in which the cysteines at positions 78 and 96 have been replaced by serines have been prepared. The resulting preparations of cytotoxic conjugates retain cytotoxic activity, are monogenous and are free of aggregates.

a. Preparation of muteins for recombinant production of the conjugates

For recombinant expression using to the methods herein, all of the cysteines of the FGF peptide that are not required for biological activity are deleted or replaced; and for use in the chemical conjugation methods herein, all except for one of these cysteines, which will be used for chemical conjugation to the cytotoxic agent ,are deleted or replaced. In practice, it appears that only two cysteines (including each of the cysteine residues set forth in Table 2), and perhaps only the cysteines set forth in Table 2, are required for retention of the requisite biological activity of the FGF peptide. Thus, FGF peptides that have more than two cysteines are modified by r placing the remaining cysteines with serines. The resulting muteins may be tested for the requisite biological activity.

FGF peptides, such as FGF-3, FGF-4 and FGF-6, that have two cysteines can be modified by replacing the second cysteine, which is not listed in Table 2, and the resulting mutein used as part of a construct containing DNA encoding the cytotoxic agent linked to the FGF-encoding DNA. The construct is expressed in a suitable host cell and the resulting protein tested for the ability to bind to FGF receptors and internalize the cytotoxic agent.

As exemplified below, conjugates containing bFGF muteins in which Cys^{78} and Cys^{96} have been replaced with serine residues have been prepared. The resulting conjugates are at least as active as recombinant conjugates that have wild type FGF components and at least as active as chemical conjugates of FGF. In addition, it appears that the recombinantly produced conjugates are less toxic, and thus, can, if necessary, be administered in higher dosages.

b. DNA constructs and expression of the DNA constructs

To produce monogenous preparations of cytotoxic conjugates using recombinant means, the DNA encoding the FGF protein is modified so that, upon expression, the resulting FGF portion of the fusion protein does not include any cysteines available for reaction. In preferred embodiments, DNA encoding an FGF polypeptide is linked to DNA encoding a saporin polypeptide. The DNA encoding the FGF polypeptide is modified in order to remove the translation stop codon and other transcriptional or translational stop signals that may be present and to remove or replace DNA encoding the available cysteines. The DNA is then ligated to the DNA encoding the saporin polypeptide directly or via a spacer region of one or more codons between the first codon of the saporin and the last codon of the FGF. The size of the spacer region is any length as long as the resulting conjugate exhibits cytotoxic activity upon internalization by a target cell. Presently, spacer regions of from about one to about seventy-five to ninety codons are preferred.

DNA encoding FGF peptides and/or the amino acid sequences FGFs are known to those of skill in this art (see, e.g., SEQ ID NOs. 24-32). DNA may be prepared synthetically based on the amino acid sequence or known DNA sequence of an FGF or may be isolated using methods known to those of skill in the art or obtained from commercial or other sources known to those of skill in this art. For example, DNA encoding virtually all of the FGF family of peptides is known. For example human aFGF (Jaye et al. (1986) Science 233:541-545), bovine bFGF (Abraham et al. (1986) Science 233:545-548), human bFGF (Abraham et al. (1986) EMBO J. 5:2523-2528; and Abraham et al. (1986) Quant. Biol. 51:657-668) and rat bFGF (see Shimasaki et al. (1988) Biochem. Biophys. Res. Comm. and Kurokawa et al. (1988) Nucleic Acids Res. 16:5201), FGF-3, FGF-7 and FGF-9 are known (see, also, U.S. Patent No. 5,155,214; U.S. Patent No. 4,956,455; U.S. Patent No. 5,026,839; and U.S. Patent No. 4,994,559, the DNASTAR database, and references discussed above and below). The amino acid sequence of an exemplary mammalian bFGF isolated from bovine pituitary tissue is also known (see, e.g., in Esch et al. (1985) Proc.Natl. Acad. Sci. USA 82:6507-6511; and U.S. Patent No. 4,956,455).

The isolated mammalian basic FGF protein is typically a 146-residue polypeptide having a molecular weight of about 16 kD, and a pI of about 9.6; it may be expressed with an amino terminal extension of about 9 residues so that the resulting protein has a molecular weight of about 18 kD.

Such DNA may then be mutagenized using standard methodologies to delete or delete and replace any cysteine residues, as describe herein, that are responsible for aggregate formation. If necessary, the identity of cysteine residues that contribute to aggregate formation may be determined empirically, by deleting and/or deleting and replacing a cysteine residue and ascertaining whether the resulting FGF with the deleted cysteine form

aggregates in solutions containing physiologically acceptable buffers and salts.

As discussed above, any FGF protein, in addition to basic FGF (bFGF) and acidic FGF (aFGF), including HST, INT/2, FGF-5, FGF-6, KGF(FGF-7), FGF-8, and FGF-9 (see, e.g., Baird et al. (1989) Brit. Med. Bull 45:438-452; Tanaka et al. (1992) Proc. Natl. Acad. Sci. USA 89:8928-8932; Miyamoto et al. (1993) Mol. Cell. Biol. 13:4251-4259; see, also, the data base, DNA* (July, 1993 release from DNASTAR, Inc. Madison, WI) for DNA and amino acid sequences of the FGF family; see SEQ ID NOs. 24-32 for amino acid sequences of FGF-1 - FGF-9, respectively), may be modified and expressed in accord with the methods herein. All of the FGF proteins induce mitogenic activity in a wide variety of normal diploid mesoderm-derived and neural crest-derived cells and this activity is mediated by binding to an FGF cell surface receptor followed by internalization. Binding to an FGF receptor followed by internalization are the activities required for an FGF protein to be suitable for use herein. A test of such "FGF mitogenic activity", which reflects the ability to bind to FGF receptors and to be internalized, is the ability to stimulate proliferation of cultured bovine aortic endothelial cells, as described in Gospodarowicz et al. (1982) J. Biol. Chem. 257:12266-12278; Gospodarowicz et al. (1976) Proc. Natl. Acad. Sci. USA 73:4120-4124.

The DNA encoding the resulting modified FGF-SAP can be inserted into a plasmid and expressed in a selected host, as described above, to produce monogenous preparations of FGF-SAP and homogeneous compositions containing monogenous FGF-SAP.

Multiple copies of the modified FGF-SAP chimera or modified FGF-cytotoxic agent chimera can be inserted into a single plasmid in operative linkage with one promoter. When expressed, the resulting protein will be an FGF-SAP multimer. Typically two to six copies of the chimera are inserted, preferably in a head to tail fashion, into one plasmid.

DNA encoding human bFGF-SAP having SEQ ID NO. 12 has been mutagenized as described in the Examples using splicing by overlap extension (SOE). Another preferred coding region is set forth in SEQ ID NO 13, nucleotides 1 - 465. In both instances, in preferred embodiments, the DNA is modified by replacing the cysteines at positions 78 and 96 with serine. The codons encoding cysteine residues at positions 78 and 96 of FGF in the FGF-SAP encoding DNA (SEQ ID NO. 12) were converted to serine codons by SOE. Each application of the SOE method uses two amplified oligonucleotide products, which have complementary ends as primers and which include an altered codon at the locus at which the mutation is desired, to produce a hybrid product. A second amplification reaction that uses two primers that anneal at the non-overlapping ends amplify the hybrid to produce DNA that has the desired alteration.

C. Properties of and use of the resulting chemical conjugates and fusion proteins

Cytotoxic conjugates agents can be prepared either by chemical conjugation, recombinant DNA technology, or combinations of recombinant expression and chemical conjugation. The methods herein are described with particular reference to bFGF and saporin. It is understood, however, that the same methods may be used to prepare and use conjugates of any member of the FGF family with SAP, modified SAP, or any other cytotoxic agent as described herein.

Using the methods and materials described above and in the Examples, chemical conjugates and fusion proteins have been synthesized. These include the following constructs:

TABLE 3

FGF CONJUGATES	
DESCRIPTION	Protein name
wild type chemical conjugate	CCFS1
mutein C78S chemical conjugate	CCFS2
mutein C96S chemical conjugate	CCFS3
mutein C96S Cys-Sap chemical conjugate	CCFS4
wild type fusion protein (FGF-Ala-Met-SAP)	FPFS1
mutein C78S fusion protein	FPFS2
mutein C96S fusion protein	FPFS3
mutein C78 & C96S fusion protein	FPFS4
wild type fusion protein (SAP-Ala-Met-FGF)	FPSF1
wild type fusion protein (FGF-Ala-Met-SAP-Ala-Met-SAP)	FPFS16

Particular details of the syntheses of the constructs are set forth in the EXAMPLES. The above constructs have been synthesized and have been or can be inserted into plasmids including pET 11 (with and without the T7 transcription terminator), pET 12 and pET 15 (NOVAGEN, Madison, WI), λpPL and pKK223-3 (PHARMACIA, P.L.) and derivatives of pKK223-3. The resulting plasmids have been and can be transformed into bacterial hosts including BL21, BL231(DE3)+pLYS S, HMS175(DE3), HMS175(DE3)+pLYS S (NOVAGEN, Madison, WI) and N4830(cI857) (see, Gottesman et al. (1980) J. Mol. Biol. 140:57-75, commercially available from PL Biochemicals, Inc, also, see, e.g., U.S. Patent Nos. 5,266,465, 5,260,223, 5,256,769, 5,256,769, 5,252,725, 5,250,296, 5,244,797, 5,236,828, 5,234,829, 5,229,273, 4,798,886, 4,849,350, 4,820,631 and 4,780,313). N4830 harbors a heavily deleted phage lambda prophage carrying the mutant c1857 temperature sensitive repressor and an active N gene.

TABLE 4

Fusion Protein Name	Plasmid(s) that Encode the Protein
FPFS1	PZ1A, PZIB, PZIC, PZID, PZIE
FPFS4	PZ2B, PZ2C
FPFS16	PZ14B
FPSF1	PZ15B

D. **Therapeutic use of the FGF conjugates**

Mouse xenograft tumor models demonstrate that the FGF conjugates exhibit anti-tumor activity. Weekly intravenous injections in mice, with established SK-Mel-5 xenografts, of wild-type bFGF-SAP conjugates (total dose 125 μg/kg) over four weeks resulted in a mean tumor volume that was 49% of the control volume. Modification of the weekly regiment to include cis-platin (5 mg/kg intraperitoneally once per week on the day following FGF-SAP treatment) resulted in a mean tumor volume at sixty days that was 23% of the controls. The combined treatment resulted in complete tumor remission in 10% of the treated mice.

Conjugates produced herein have been injected into such mice and appear to be less toxic than heterogeneous preparations of chemical conjugates. Certain of the conjugates provided herein have also been shown to exhibit anti-tumor activity in such mice.

In particular 5 μg/kg/week of FPFS1 and CCFS1 were administered to mice, with established HT-1197 (a human bladder carcinoma cell line) xenografts. Each treatment resulted in significant inhibition of tumor growth throughout the 61 days of the study. In another study, 0.1 or 0.5 μg/kg/week of FPFS1 with and without 0.5 mg/kg cisplatin is administered to mice with established human prostate carcinoma cell tumors.

The chemical conjugate and fusion protein bFGF-SAP provided herein may also be used for the treatment of restenosis. FGF conjugates have an anti-proliferative effect on smooth muscle cells in rabbit balloon injury models of restenosis (see, also U.S. Patent No. 5,308,622, which is based

on allowed U.S. Application Serial No. 07/915,056, which describes the use of FGF-cytotoxic conjugates for the treatment of restenosis).

E. Formulation and administration of pharmaceutical compositions

The conjugates herein may be formulated into pharmaceutical compositions suitable for topical, local, intravenous and systemic application. Effective concentrations of one or more of the conjugates are mixed with a suitable pharmaceutical carrier or vehicle. The concentrations or amounts of the conjugates that are effective requires delivery of an amount, upon administration, that ameliorates the symptoms or treats the disease. Typically, the compositions are formulated for single dosage administration. Therapeutically effective concentrations and amounts may be determined empirically by testing the conjugates in known in vitro and in vivo systems, such as those described here; dosages for humans or other animals may then be extrapolated therefrom.

Upon mixing or addition of the conjugate(s) with the vehicle, the resulting mixture may be a solution, suspension, emulsion or the like. The form of the resulting mixture depends upon a number of factors, including the intended mode of administration and the solubility of the conjugate in the selected carrier or vehicle. The effective concentration is sufficient for ameliorating the symptoms of the disease, disorder or condition treated and may be empirically determined based upon in vitro and/or in vivo data, such as the data from the mouse xenograft model. If necessary, pharmaceutically acceptable salts or other derivaives of the conjugates may be prepared.

Pharmaceutical carriers or vehicles suitable for administration of the conjugates provided herein include any such carriers known to those skilled in the art to be suitable for the particular mode of administration.

In addition, the conjugates may be formulated as the sole pharmaceutically active ingredient in the composition or may be combined with other active ingredients.

The conjugates can be administered by any appropriate route, for example, orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid, semi-liquid or solid form and are formulated in a manner suitable for each route of administration. Preferred modes of administration depend upon the indication treated. Dermatological and ophthalmologic indications will typically be treated locally; whereas, tumors and restenosis, will typically be treated by systemic, intradermal or intramuscular, modes of administration.

The conjugate is included in the pharmaceutically acceptable carrier in an amount sufficient to exert a therapeutically useful effect in the absence of undesirable side effects on the patient treated. It is understood that the number and degree of side effects depends upon the condition for which the conjugates are administered. For example, certain toxic and undesirable side effects are tolerated when treating life-threatening illnesses, such as tumors, that would not be tolerated when treating disorders of lesser consequence.

The concentration of conjugate in the composition will depend on absorption, inactivation and excretion rates thereof, the dosage schedule, and amount administered as well as other factors known to those of skill in the art.

Typically a therapeutically effective dosage should produce a serum concentration of active ingredient of from about 0.1 ng/ml to about 50-100 μg/ml. The pharmaceutical compositions typically should provide a dosage of from about 0.01 mg to about 100 - 2000 mg of conjugate, depending upon the conjugate selected, per kilogram of body weight per day. For example, for treatment of restenosis a daily dosage of about between 0.05 and 0.5 mg/kg (based on FGF-SAP chemical conjugate or an amount of conjugate provided herein equivalent on a molar basis thereto) should be sufficient. It is understood that the amount to administer will be a function of the conjugate selected, the indication treated, and possibly the side effects that will be tolerated.

The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at intervals of time. It is understood that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions.

Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include any of the following components: a sterile diluent, such as water for injection, saline solution, fixed oil, polyethylene glycol, glycerine, propylene glycol or other synthetic solvent; antimicrobial agents, such as benzyl alcohol and methyl parabens; antioxidants, such as ascorbic acid and sodium bisulfite; chelating agents, such as ethylenediaminetetraacetic acid (EDTA); buffers, such as acetates, citrates and phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. Parental preparations can be enclosed in ampules, disposable syringes or multiple dose vials made of glass, plastic or other suitable material.

If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and solutions containing thickening and solubilizing agents, such as glucose, polyethylene glycol, and polypropylene glycol and mixtures thereof. Liposomal suspensions may also be suitable as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art.

The conjugates may be prepared with carriers that protect them against rapid elimination from the body, such as time release formulations or coatings. Such carriers include controlled release formulations, such as, but not limited to, implants and microencapsulated delivery systems, and biodegradable, biocompatible polymers, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, polyorthoesters, polylactic acid and others. Methods for preparation of such formulations are known to those skilled in the art.

The conjugates may be formulated for local or topical application, in the form of gels, creams, and lotions and for intracisternal or intraspinal application. Such solutions may be formulated as 0.01% -10% isotonic solutions, pH about 5-7, with appropriate salts. The conjugates may be formulated as aerosols for topical application (see, e.g., U.S. Patent Nos. 4,044,126, 4,414,209, and 4,364,923).

If oral administration is desired, the conjugate should be provided in a composition that protects it from the acidic environment of the stomach. For example, the composition can be formulated in an enteric coating that maintains its integrity in the stomach and releases the active compound in the intestine. The composition may also be formulated in combination with an antacid or other such ingredient.

Oral compositions will generally include an inert diluent or an edible carrier and may be compressed into tablets or enclosed in gelatin capsules. For the purpose of oral therapeutic administration, the active compound or compounds can be incorporated with excipients and used in the form of tablets, capsules or troches. Pharmaceutically compatible binding agents and adjuvant materials can be included as part of the composition.

The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder, such as microcrystalline cellulose, gum tragacanth and gelatin; an excipient such as starch and lactose, a disintegrating agent such as, but not limited to, alginic acid and corn starch; a lubricant such as, but not limited to,

magnesium stearate; a glidant, such as, but not limited to, colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; and a flavoring agent such as peppermint, methyl salicylate, and fruit flavoring.

When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar and other enteric agents. The conjugates can also be administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors.

The active materials can also be mixed with other active materials that do not impair the desired action, or with materials that supplement the desired action, such as cis-platin for treatment of tumors.

Finally, the compounds may be packaged as articles of manufacture containing packaging material, one or more conjugates or compositions as provided herein within the packaging material, and a label that indicates the indication for which the conjugate is provided.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

RECOMBINANT PRODUCTION OF SAPORIN

A. Materials and methods

1. Bacterial Strains:

E. coli strain JA221 (lpp⁻ hdsM+ trpE5 leuB6 lacY recA1 F'[lacI^q lac$^+$ pro$^+$]) is publicly available from the American Type Culture Collection (ATCC), Rockville, MD 20852, under the accession number ATCC 33875. (JA221 is also available from the Northern Regional Research Center (NRRL), Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604, under the accession number NRRL B-15211; see, also, U.S. Patent No. 4,757,013 to Inouye; and Nakamura et al. (1979) Cell 18:1109-1117.) Strain INV1α is commercially available from Invitrogen, San Diego, CA.

2. DNA Manipulations

The restriction and modification enzymes employed herein are commercially available in the U.S. Native saporin and rabbit polyclonal antiserum to saporin were obtained as previously described in Lappi et al. (1985) Biochem. Biophys. Res. Comm. 129:934-942. Ricin A chain is commercially available from SIGMA, Milwaukee, WI. Antiserum was linked to Affi-gel 10 (BIO-RAD, Emeryville, CA) according to the manufacturer's instructions. Sequencing was performed using the Sequenase kit of United States Biochemical Corporation (version 2.0) according to the manufacturer's instructions. Minipreparation and maxipreparations of plasmids, preparation of competent cells, transformation, M13 manipulation, bacterial media, Western blotting, and ELISA assays were according to Sambrook et al. ((1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The purification of DNA fragments was done using the Geneclean II kit (Bio 101) according to the manufacturer's instructions. SDS gel electrophoresis was performed on a Phastsystem (Pharmacia).

Western blotting was accomplished by transfer of the electrophoresed protein to nitrocellulose using the PhastTransfer system, as described by the manufacturer. The antiserum to SAP was used at a dilution of 1:1000. Horseradish peroxidase labelled anti-IgG was used as the second antibody (see Davis et al. (1986) Basic methods in molecular biology, New York, Elsevier Science Publishing Co., pp 1-338).

B. Isolation of DNA encoding saporin

1. Isolation of genomic DNA and preparation of polymerase chain reaction (PCR) primers

Saponaria officinalis leaf genomic DNA was prepared as described in Bianchi et al. (1988) Plant Mol. Biol. 11:203-214. Primers for genomic DNA amplifications were synthesized in a 380B automatic DNA synthesizer. The primer corresponding to the "sense" strand of saporin (SEQ ID NO 1) includes an EcoR I restriction site adapter immediately upstream of the DNA codon for amino acid -15 of the native saporin N-terminal leader sequence (SEQ ID NO. 1):

5'-CTGCAGAATTCGCATGGATCCTGCTTCAAT-3'.

The primer 5'-CTGCAGAATTCGCCTCGTTTGACTACTTTG-3' (SEQ ID NO. 2) corresponds to the "antisense" strand of saporin and complements the coding sequence of saporin starting from the last 5 nucleotides of the DNA encoding the carboxyl end of the mature peptide. Use of this primer introduced a translation stop codon and an EcoRI restriction site after the sequence encoding mature saporin.

2. Amplification of DNA encoding saporin

Unfractionated Saponaria officinalis leaf genomic DNA (1 μl) was mixed in a final volume of 100 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 μg of each primer. Next, 2.5 U TaqI DNA polymerase (Perkin Elmer Cetus) was added and the mixture was overlaid with 30 μl of mineral oil (Sigma). Incubations were done in a DNA Thermal Cycler (Perkin Elmer Cetus). One cycle included a denaturation step (94°C for 1 min.), an annealing step (60°C for

2 min.), and an elongation step (72°C for 3 min.). After 30 cycles, a 10 μl aliquot of each reaction was run on a 1.5% agarose gel to verify the correct structure of the amplified product.

The amplified DNA was digested with EcoRI and subcloned into EcoR I-restricted M13mp18 (NEW ENGLAND BIOLABS, Beverly, MA; see, also, Yanisch-Perron et al. (1985), "Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors", Gene 33:103). Single-stranded DNA from recombinant phages was sequenced using oligonucleotides based on internal points in the coding sequence of saporin (see, Bennati et al. (1989) Eur. J. Biochem. 183:465-470). Nine of the M13mp18 derivatives were sequenced and compared. Of the nine sequenced clones, five had unique sequences, set forth as SEQ ID NOs 3-7, respectively. The clones were designated M13mp18-G4, -G1, -G2, -G7, and -G9. Each of these clones contains all of the saporin coding sequence and 45 nucleotides of DNA encoding the native saporin N-terminal leader peptide.

C. pOMPAG4 Plasmid Construction

M13 mp18-G4, containing the SEQ ID NO. 3 clone from Example 1.B.2., was digested with EcoR I, and the resulting fragment was ligated into the EcoR I site of the vector pIN-IIIompA2 (see, e.g., U.S. Patent No. 4,575,013 to Inouye; and Duffaud et al. (1987) Meth. Enz. 153:492-507) using the methods described in Example 1.A.2. The ligation was accomplished such that the DNA encoding saporin, including the N-terminal extension, was fused to the leader peptide segment of the bacterial ompA gene. The resulting plasmid pOMPAG4 contains the lpp promoter [Nakamura, K. and Inouye, M. Cell., 18:1109-1117 (1979)], the E. coli lac promoter operator sequence (lac O) and the E. coli ompA gene secretion signal in operative association with each other and with the saporin and native N-terminal leader-encoding DNA listed in SEQ ID NO. 3. The plasmid also includes the E. coli lac repressor gene (lac I).

The M13 mp18-G1, -G2, -G7, and -G9 clones obtained from Example 1.B.2, containing SEQ ID NOs. 4-7 respectively, are digested with EcoR I and ligated into EcoR I digested pIN-IIIompA2 as described for M13 mp18-G4 above in this example. The resulting plasmids, labeled pOMPAG1, pOMPAG2, pOMPAG7, pOMPA9, are screened, expressed, purified, and characterized as described for the plasmid pOMPAG4.

INV1α competent cells were transformed with pOMPAG4 and cultures containing the desired plasmid structure were grown further in order to obtain a large preparation of isolated pOMPAG4 plasmid using methods described in Example 1.A.2.

D. Saporin expression in E. coli:

The pOMPAG4 transformed E. coli cells were grown under conditions in which the expression of the saporin-containing protein is repressed by the lac repressor to an O.D. in or at the end of the log phase of growth after which IPTG was added to induce expression of the saporin-encoding DNA.

To generate a large-batch culture of pOMPAG4 transformed E. coli cells, an overnight culture (lasting approximately 16 hours) of JA221 E. coli cells transformed with the plasmid pOMPAG4 in LB broth (see e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) containing 125 mg/ml ampicillin was diluted 1:100 into a flask containing 750 ml LB broth with 125 mg/ml ampicillin. Cells were grown at logarithmic phase shaking at 37°C until the optical density at 550 nm reached 0.9 measured in a spectrophotometer.

In the second step, saporin expression was induced by the addition of IPTG (Sigma) to a final concentration of 0.2 mM. Induced cultures were grown for 2 additional hours and then harvested by centrifugation (25 min., 6500 x g). The cell pellet was resuspended in ice cold 1.0 M TRIS, pH 9.0, 2 mM EDTA (10 ml were added to each gram of pellet). The resuspended material was kept on ice for 20-60 minutes and then

centrifuged (20 min., 6500 x g) to separate the periplasmic fraction of E. coli, which corresponds to the supernatant, from the intracellular fraction corresponding to the pellet.

E. Purification of secreted recombinant Saporin

1. Anti-SAP immuno-affinity purification

The periplasmic fraction from Example 1.D. was dialyzed against borate-buffered saline (BBS: 5 mM boric acid, 1.25 mM borax, 145 mM sodium chloride, pH 8.5). The dialysate was loaded onto an immunoaffinity column (0.5 x 2 cm) of anti-saporin antibodies, obtained as described in Lappi et al., Biochem. Biophys. Res. Comm., 129: 934-942 (1985), bound to Affi-gel 10 and equilibrated in BBS at a flow rate of about 0.5 ml/min. The column was washed with BBS until the absorbance at 280 nm of the flow-through was reduced to baseline. Next the column containing the antibody bound saporin was eluted with 1.0 M acetic acid and 0.5 ml fractions were collected in tubes containing 0.3 ml of 2 M ammonium hydroxide, pH 10. The fractions were analyzed by ELISA (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The peak fraction of the ELISA was analyzed by Western blotting as described in Example 1.A.2 and showed a single band with a slightly higher molecular weight than native saporin. The fractions that contained saporin protein, as determined by the ELISA, were then pooled for further purification.

2. Reverse Phase High Performance Liquid Chromatography purification

To further purify the saporin secreted into the periplasm, the pooled fractions from Example 1.E.1. were diluted 1:1 with 0.1% trifluoroacetic acid (TFA) in water and chromatographed in reverse phase high pressure liquid chromatography (HPLC) on a Vydac C4 column (Western Analytical) equilibrated in 20% acetonitrile, 0.1% TFA in water. The protein was eluted with a 20 minute gradient to 60% acetonitrile. The HPLC produced a single peak that was the only area of immunoreactivity with anti-SAP

antiserum when analyzed by a western blot as described in Example 1.E.1. Samples were assayed by an ELISA.

Sequence analysis was performed by Edman degradation in a gas-phase sequenator (Applied Biosystems) (see, e.g., Lappi et al. (1985) Biochem. Biophys. Res. Comm.129:934-942). The results indicated that five polypeptides were obtained that differ in the length, between 7 and 12 amino acids, of the N-terminal saporin leader before the initial amino acid valine of the mature native saporin (SEQ ID NO 3: residue -12 through -7). All of the N-terminal extended variants retained cytotoxic activity. The size of the native leader is 18 residues, indicating that the native signal peptide is not properly processed by bacterial processing enzymes. The ompA signal was, however, properly processed.

To obtain homogeneous saporin, the recombinantly produced saporin can be separated by size and one of the five polypeptides used to produce the conjugates.

F. Purification of intracellular soluble saporin

To purify the cytosolic soluble saporin protein, the pellet from the intracellular fraction of Example 1.E. above was resuspended in lysis buffer (30 mM TRIS, 2 mM EDTA, 0.1% Triton X-100, pH 8.0, with 1 mM PMSF, 10 μg/ml pepstatin A, 10 μg aprotinin, μg/ml leupeptin and 100 μg/ml lysozyme, 3.5 ml per gram of original pellet). To lyse the cells, the suspension was left at room temperature for one hour, then frozen in liquid nitrogen and thawed in a 37°C bath three times, and then sonicated for two minutes. The lysate was centrifuged at 11,500 x g for 30 min. The supernatant was removed and stored. The pellet was resuspended in an equal volume of lysis buffer, centrifuged as before, and this second supernatant was combined with the first. The pooled supernatants were dialyzed versus BBS and chromatographed over the immunoaffinity column as described in Example 1.E.1. This material also retained cytotoxic activity.

G. **Assay for cytotoxic activity**

The RIP activity of recombinant saporin was compared to the RIP activity of native SAP in an in vitro assay measuring cell-free protein synthesis in a nuclease-treated rabbit reticulocyte lysate (Promega). Samples of immunoaffinity-purified saporin, obtained in Example 1.E.1., were diluted in PBS and 5 μl of sample was added on ice to 35 μl of rabbit reticulocyte lysate and 10 μl of a reaction mixture containing 0.5 μl of Brome Mosaic Virus RNA, 1 mM amino acid mixture minus leucine, 5 μCi of tritiated leucine and 3 μl of water. Assay tubes were incubated 1 hour in a 30°C water bath. The reaction was stopped by transferring the tubes to ice and adding 5 μl of the assay mixture, in triplicate, to 75 μl of 1 N sodium hydroxide, 2.5% hydrogen peroxide in the wells of a Millititer HA 96-well filtration plate (Millipore). When the red color had bleached from the samples, 300 μl of ice cold 25% trichloroacetic acid (TCA) were added to each well and the plate left on ice for another 30 min. Vacuum filtration was performed with a Millipore vacuum holder. The wells were washed three times with 300 μl of ice cold 8% TCA. After drying, the filter paper circles were punched out of the 96-well plate and counted by liquid scintillation techniques.

The IC_{50} for the recombinant and native saporin were approximately 20 pM. Therefore, recombinant saporin-containing protein has full protein synthesis inhibition activity when compared to native saporin.

EXAMPLE 2

RECOMBINANT PRODUCTION OF FGF-SAP FUSION PROTEIN

A. **General Descriptions**

1. **Bacterial Strains and Plasmids**

E. coli strains BL21(DE3), BL21(DE3)pLysS, HMS174(DE3) and HMS174(DE3)pLysS were purchased from NOVAGEN, Madison, WI. Plasmid pFC80, described below, has been described in the WIPO International Patent Application No. WO 90/02800, except that the bFGF coding sequence in the plasmid designated pFC80 herein has the sequence

set forth as SEQ ID NO 12, nucleotides 1-465. The plasmids described herein may be prepared using pFC80 as a starting material or, alternatively, by starting with a fragment containing the CII ribosome binding site (SEQ ID NO 15) linked to the FGF-encoding DNA (SEQ ID NO 12).

2. DNA Manipulations

The restriction and modification enzymes employed here are commercially available in the U.S. Native SAP, chemically conjugated bFGF-SAP and rabbit polyclonal antiserum to SAP and FGF were obtained as described in Lappi et al., Biochem. Biophys. Res. Comm., 129: 934-942 (1985) and Lappi et al., Biochem. Biophys., Res. Comm., 160: 917-923 (1989). The pET System Induction Control was purchased from NOVAGEN, Madison, WI. The sequencing of the different constructions was done using the Sequenase kit of United States Biochemical Corporation (version 2.0). Minipreparation and maxipreparations of plasmids, preparation of competent cells, transformation, M13 manipulation, bacterial media and Western blotting were performed using routine methods (see, e.g.,.Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The purification of DNA fragments was done using the Geneclean II kit, purchased from Bio 101. SDS gel electrophoresis was performed on a Phastsystem (Pharmacia).

B. Construction of plasmids encoding FGF-SAP fusion proteins

1. Construction of FGFM13 that contains DNA encoding the CI ribosome binding site linked to FGF

A Nco I restriction site was introduced into the SAP-encoding DNA the M13mp18-G4 clone, prepared as described in Example 1.B.2. by site-directed mutagenesis method using the Amersham In vitro-mutagenesis system 2.1. The oligonucleotide employed to create the Nco I restriction site was synthesized using a 380B automatic DNA synthesizer (Applied Biosyst ms) and is listed as:

SEQ ID NO 8 - CAACAACTGCCATGGTCACATC.

This oligonucleotide containing the Nco I site replaced the original SAP-containing coding sequence at SEQ ID NO 3, nts 32-53. The resulting M13mp18-G4 derivative is termed mpNG4.

In order to produce a bFGF coding sequence in which the stop codon was removed, the FGF-encoding DNA was subcloned into a M13 phage and subjected to site-directed mutagenesis. Plasmid pFC80 is a derivative of pDS20 (see, e.g., Duester et al. (1982) Cell 30:855-864; see also U.S. Patent Nos. 4,914,027, 5,037,744, 5,100,784, and 5,187,261; see, also, PCT International Application No. WO 90/02800; and European Patent Application No. EP 267703 A1), which is almost the same as plasmid pKG1800 (see, Bernardi et al. (1990) DNA Sequence 1:147-150; see, also McKenney et al. (1981) pp. 383-415 in Gene Amplification and Analysis 2: Analysis of Nucleic Acids by Enzymatic Methods Chirikjian et al., eds, North Holland Publishing Company, Amsterdam) except that it contains an extra 440 bp at the distal end of galK between nucleotides 2440 and 2880 in pDS20. Plasmid pKG1800 includes the 2880 bp EcoR I-Pvu II of pBR322 that contains the contains the ampicillin resistance gene and an origin of replication.

Plasmid pFC80 was prepared from pDS20 by replacing the entire galK gene with the FGF-encoding DNA of SEQ ID NO. 12, inserting the trp promoter (SEQ ID NO. 14) and the bacteriophage lambda CII ribosome binding site (SEQ. ID No. 15; see, e.g., Schwarz et al. (1978) Nature 272:410) upstream of and operatively linked to the FGF-encoding DNA. The Trp promoter can be obtained from plasmid pDR720 (Pharmacia PL Biochemicals) or synthesized according to SEQ ID NO. 14. Plasmid pFC80, contains the 2880 bp EcoR I-BamH I fragment of plasmid pSD20, a synthetic Sal I-Nde I fragment that encodes the Trp promoter region (SEQ ID NO. 14):

EcoR I
AATTCCCCTGTTGACAATTAATCATCGAACTAGTTAACTAGTACGCAGCTTGGCTGCAG

and the CII ribosome binding site (SEQ ID NO.15)):

Sal I Nde I
GTCGACCAAGCTTGGGCATACATTCAATCAATTGTTATCTAAGGAAATACTTACATATG

The FGF-encoding DNA was removed from pFC80 by treating it as follows. The pFC80 plasmid was digested by Hga I and Sal I, which produces a fragment containing the CII ribosome binding site linked to the FGF-encoding DNA. The resulting fragment was blunt ended with Klenow's reagent and inserted into M13mp18 that had been opened by Sma I and treated with alkaline phosphatase for blunt-end ligation. In order to remove the stop codon, an insert in the ORI minus direction was mutagenized using the Amersham kit, as described above, using the following oligonucleotide (SEQ ID NO 9): GCTAAGAGCGCCATGGAGA. SEQ ID NO 9 contains 1 nucleotide between the FGF carboxy terminal serine codon and a Nco I restriction site, and it replaced the following wild type FGF encoding DNA having SEQ ID NO 10:

```
GCT AAG AGC TGA  CCA TGG AGA.
Ala Lys Ser STOP Pro Trp Arg
```

The resulting mutant derivative of M13mp18, lacking a native stop codon after the carboxy terminal serine codon of bFGF, was designated FGFM13. The mutagenized region of FGFM13 contained the correct sequence (SEQ ID NO 11).

2. Preparation of plasmids pFS92 (PZ1A), PZ1B and PZ1C that encode the FGF-SAP fusion protein

a. Plasmid pFS92 (also designated PZ1A)

Plasmid FGFM13 was cut with Nco I and Sac I to yield a fragment containing the CII ribosome binding site linked to the bFGF coding sequence with the stop codon replaced.

The M13mp18 derivative mpNG4 containing the saporin coding sequence was also cut with restriction endonucleases Nco I and Sac I, and the bFGF coding fragment from FGFM13 was inserted by ligation to DNA encoding the fusion protein bFGF-SAP into the M13mp18 derivative to produce mpFGF-SAP, which contains the CII ribosom binding site linked to the FGF-SAP fusion gene. The sequence of the fusion gene is set forth in

SEQ ID NO 12 and indicates that the FGF protein carboxy terminus and the saporin protein amino terminus are separated by 6 nucleotides (SEQ ID NOs 12 and 13, nts 466-471) that encode two amino acids Ala Met.

Plasmid mpFGF-SAP was digested with Xba I and EcoR I and the resulting fragment containing the bFGF-SAP coding sequence was isolated and ligated into plasmid pET-11a (available from NOVAGEN, Madison, WI; for a description of the plasmids see U.S. Patent No. 4,952,496; see, also Studier et al. (1990) Meth. Enz. 185:60-89; Studier et al. (1986) J. Mol. Biol. 189:113-130; Rosenberg et al. (1987) Gene 56:125-135) that had also been treated with EcoR I and Xba I. The resulting plasmid was designated pFS92. It was renamed PZ1A.

Plasmid pFS92 (or PZ1A) contains DNA the entire basic FGF protein (SEQ ID NO 12), a 2-amino acid long connecting peptide, and amino acids 1 to 253 of the mature SAP protein. Plasmid pFS92 also includes the CII ribosome binding site linked to the FGF-SAP fusion protein and the T7 promoter region from pET-11a.

E. coli strain BL21(DE3)pLysS (NOVAGEN, Madison WI) was transformed with pFS92 according to manufacturer's instructions and the methods described in Example 2.A.2.

b. Plasmid PZ1B

Plasmid pFS92 was digested with EcoR I, the ends repaired by adding nucleoside triphosphates and Klenow DNA polymerase, and then digested with Nde I to release the FGF-encoding DNA without the CII ribosome binding site. This fragment was ligated into pET 11a, which had been BamH I digested, treated to repair the ends, and digested with Nde I. The resulting plasmid was designated PZ1B. PZ1B includes the T7 transcription terminator and the pET-11a ribosome binding site.

E. coli strain BL21(DE3) (NOVAGEN, Madison WI) was transformed with PZ1B according to manufacturer's instructions and the methods described in Example 2.A.2.

c. Plasmid PZ1C

Plasmid PZ1C was prepared from PZ1B by replacing the ampicillin resistance gene with a kanamycin resistance gene.

d. Plasmid PZ1D

Plasmid pFS92 was digested with EcoR I and Nde I to release the FGF-encoding DNA without the CII ribosome binding site and the ends were repaired. This fragment was ligated into pET 12a, which had been BamH I digested and treated to repair the ends. The resulting plasmid was designated PZ1D. PZ1D includes DNA encoding the OMP T secretion signal operatively linked to DNA encoding the fusion protein.

E. coli strains BL21(DE3), BL21(DE3)pLysS, HMS174(DE3) and HMS174(DE3)pLysS (NOVAGEN, Madison WI) were transformed with PZ1D according to manufacturer's instructions and the methods described in Example 2.A.2.

C. Expression of the recombinant bFGF-SAP fusion proteins

The two-stage method described above was used to produce recombinant bFGF-SAP protein (hereinafter bFGF-SAP fusion protein).

1. Expression of rbFGF-SAP from pFS92 (PZ1A)

Three liters of LB broth containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml) were inoculated with pFS92 plasmid-containing bacterial cells (strain BL21(DE3)pLysS) from an overnight culture (1:100 dilution) that were obtained according to Example 2.B. Cells were grown at 37° C in an incubator shaker to an OD_{600} of 0.7. IPTG (Sigma Chemical, St. Louis, MO) was added to a final concentration of 0.2 mM and growth was continued for 1.5 hours at which time cells were centrifuged. Subsequent experiments have shown that growing the BL21(DE3)pLysS cells at 30° C instead of 37° C improves yields. When the cells are grown at 30° C they are grown to an OD_{600} of 1.5 prior to induction. Following induction, growth is continued for about 2 to 2.5 hours at which time the cells are harvested by centrifugation.

The pellet was resuspended in lysis solution (45-60 ml per 16 g of pellet; 20 mM TRIS, pH 7.4, 5 mM EDTA, 10% sucrose, 150 mM NaCl, lysozyme, 100 μg/ml, aprotinin, 10 μg/ml, leupeptin, 10 μg/ml, pepstatin A, 10 μg/ml and 1 mM PMSF) and incubated with stirring for 1 hour at room temperature. The solution was frozen and thawed three times and sonicated for 2.5 minutes. The suspension was centrifuged at 12,000 X g for 1 hour; the resulting first-supernatant was saved and the pellet was resuspended in another volume of lysis solution without lysozyme. The resuspended material was centrifuged again to produce a second-supernatant, and the two supernatants were pooled and dialyzed against borate buffered saline, pH 8.3.

2. Expression of bFGF-SAP fusion protein from PZ1B and PZ1C

Two hundred and fifty mls. of LB medium containing ampicillin (100 μg/ml) were inoculated with a fresh glycerol stock of PZ1B. Cells were grown at 30° C in an incubator shaker to an OD_{600} of 0.7 and stored overnight at 4° C. The following day the cells were pelleted and resuspended in fresh LB medium (no ampicillin). The cells were divided into 5 1-liter batches and grown at 30° C in an incubator shaker to an OD_{600} of 1.5. IPTG (SIGMA CHEMICAL, St. Louis, MO) was added to a final concentration of 0.1 mM and growth was continued for about 2 to 2.5 hours at which time cells were harvested by centrifugation.

In order to grow PZ1C, prior to induction, the cells are grown in medium containing kanamycin (50μg/ml) in place of ampicillin.

3. Expression of bFGF-SAP fusion protein from PZ1D

Two hundred and fifty mls of LB medium containing ampicillin (100 μg/ml) were inoculated with a fresh glycerol stock of PZ1B. Cells were grown at 30° C in an incubator shaker to an OD_{600} of 0.7 and stored overnight at 4° C. The following day the cells were pelleted and resuspended in fresh LB medium (no ampicillin). The cells were used to inoculate a 1 liter batch of LB medium and grown at 30° C in an incubator

shaker to an OD_{600} of 1.5. IPTG (SIGMA CHEMICAL, St. Louis, MO) was added to a final concentration of 0.1 mM and growth was continued for about 2 to 2.5 hours at which time cells were harvested by centrifugation.

The cell pellet was resuspended in ice cold 1.0 M Tris pH 9.0. 2 mM EDTA. The resuspended material is kept on ice for another 20-60 minutes and then centrifuged to separate the periplasmic fraction (supernatant) from the intracellular fraction (pellet).

D. Affinity purification of bFGF-SAP fusion protein

Thirty ml of the dialyzed solution containing the bFGF-SAP fusion protein from Example 2.C. was applied to HiTrap heparin-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with 0.15 M NaCl in 10 mM TRIS, pH 7.4 (buffer A). The column was washed: first with equilibration buffer; second with 0.6 M NaCl in buffer A; third with 1.0 M NaCl in buffer A; and finally eluted with 2 M NaCl in buffer A into 1.0 ml fractions. Samples were assayed by the ELISA method.

The results indicate that the bFGF-SAP fusion protein elutes from the heparin-Sepharose column at the same concentration (2 M NaCl) as native and recombinantly-produced bFGF. This indicates that the heparin affinity is retained in the bFGF-SAP fusion protein.

E. Characterization of the bFGF-SAP fusion protein

1. Western blot of affinity-purified bFGF-SAP fusion protein

SDS gel electrophoresis was performed on a Phastsystem utilizing 20% gels (Pharmacia). Western blotting was accomplished by transfer of the electrophoresed protein to nitrocellulose using the PhastTransfer system (Pharmacia), as described by the manufacturer. The antisera to SAP and bFGF were used at a dilution of 1:1000. Horseradish peroxidase labeled anti-IgG was used as the second antibody (Davis et al. (1986) Basic Methods in Molecular Biology, New York, Elsevier Science Publishing Co., pp 1-338).

The anti-SAP and anti-FGF antisera bound to a protein with an approximate molecular weight of 48,000 kd, which corresponds to the sum of the independent molecular weights of SAP (30,000) and bFGF (18,000).

2. Assays to assess the cytotoxicity of the FGF-SAP fusion protein

a. Effect of bFGF-SAP fusion protein on cell-free protein synthesis

The RIP activity of bFGF-SAP fusion protein compared to the FGF-SAP chemical conjugate was assayed as described in Example 1.G. The results indicated that the IC_{50} of the bFGF-SAP fusion protein is about 0.2 nM and the IC_{50} of chemically conjugated FGF-SAP is about 0.125 nm.

b. Cytotoxicity of bFGF-SAP fusion protein

Cytotoxicity experiments were performed with the Promega (Madison, WI) CellTiter 96 Cell Proliferation/Cytotoxicity Assay. About 1,500 SK-Mel-28 cells (available from ATCC), a human melanoma cell line, were plated per well in a 96 well plate in 90 μl HDMEM plus 10% FCS and incubated overnight at 37°C, 5% CO_2. The following morning 10 μl of media alone or 10 μl of media containing various concentrations of the rbFGF-SAP fusion protein, basic FGF or saporin were added to the wells. The plate was incubated for 72 hours at 37°C. Following the incubation period, the number of living cells was determined by measuring the incorporation and conversion of the commonly available dye MTT supplied as a part of the Promega kit. Fifteen μl of the MTT solution was added to each well, and incubation was continued for 4 hours. Next, 100 μl of the standard solubilization solution supplied as a part of the Promega kit was added to each well. The plate was allowed to stand overnight at room temperature and the absorbance at 560 nm was read on an ELISA plate reader (Titertek Multiskan PLUS, ICN, Flow, Costa Mesa, CA).

The results indicated that the chemical FGF-SAP conjugate has an ID_{50} of 0.3 nM, the bFGF-SAP fusion protein has a similar ID_{50} of 0.6 nM, and unconjugated SAP, which is unable to bind to the cell surface, has an ID_{50} of 200 nM. Therefore, when internalized, the bFGF-SAP fusion

protein appears to have approximately the same cytotoxic activity as the chemically conjugated FGF-SAP.

EXAMPLE 3

PREPARATION OF FGF MUTEINS

A. Materials and Methods

1. Reagents

Restriction and modification enzymes were purchased from BRL (Gaithersburg, MD), Stratagene (La Jolla, CA) and New England Biolabs (Beverly, MA). Native SAP, chemically conjugated basic FGF-SAP and rabbit polyclonal antiserum to SAP and basic FGF were obtained from Saponaria officinalis (see, e.g., Stirpe et al. (1983) Biochem. J. 216:617-625). Briefly, the seeds were extracted by grinding in 5 mM sodium phosphate buffer, pH 7.2 containing 0.14 M NaCl, straining the extracts through cheesecloth, followed by centrifugation at 28,000 g for 30 min to produce a crude extract, which was dialyzed against 5 mM sodium phosphate buffer, pH 6.5, centrifuged and applied to CM-cellulose (CM 52, Whatman, Maidstone, Kent, U.K.). The CM column was washed and SO-6 was eluted with a 0-0.3 M NaCl gradient in the phosphate buffer.

Plasmid pFC80, containing the basic FGF coding sequence, was a gift of Drs. Paolo Sarmientos and Antonella Isacchi of Farmitalia Carlo Erba (Milan, Italy). Plasmid pFC80, has been described in WIPO International Patent Application No. WO 90/02800 and co-pending International PCT Application Serial No. PCT/US93/05702 (published as WO 93/25688), which are herein incorporated in their entirety by reference. The sequence of DNA encoding bFGF in pFC80 is that set forth in copending International PCT Application Serial No. PCT/US93/05702 and in SEQ ID NO. 12. The construction of pFC80 is set forth above in Example 2.

Plasmid isolation, production of competent cells, transformation and M13 manipulations were carried out according to published procedures (Sambrook et al. (1989) *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Purification of

DNA fragments was achieved using the Geneclean II kit, purchased from Bio 101 (La Jolla, CA). Sequencing of the different constructions was performed using the Sequenase kit (version 2.0) of USB (Cleveland, OH).

2. Sodium dodecyl sulphate (SDS) gel electrophoresis and Western blotting.

SDS gel electrophoresis was performed on a PhastSystem utilizing 20% gels (Pharmacia). Western blotting was accomplished by transfer of electrophoresed protein to nitrocellulose using the PhastTransfer system (Pharmacia), as described by the manufacturer. The antisera to SAP and basic FGF were used at a dilution of 1:1000. Horseradish peroxidase labeled anti-IgG was used as the second antibody as described (Davis, L., Dibner et al. (1986) *Basic Methods in Molecular Biology*, p. 1, Elsevier Science Publishing Co., New York).

B. Preparation of the mutagenized FGF by site-directed mutagenesis

Cysteine to serine substitutions were made by oligonucleotide-directed mutagenesis using the Amersham (Arlington Heights, IL) in vitro-mutagenesis system 2.1. Oligonucleotides encoding the new amino acid were synthesized using a 380B automatic DNA synthesizer (Applied Biosystems, Foster City, CA).

1. mutagenesis

The oligonucleotide used for in vitro mutagenesis of cysteine 78 was AGGAGTGTCTGCTAACC (SEQ ID NO. 16), which spans nucleotides 225-241 of SEQ ID NO. 12. The oligonucleotide for mutagenesis of cysteine 96 was TTCTAAATCGGTTACCGATGACTG (SEQ ID NO. 17), which spans nucleotides 279-302 of SEQ ID NO. 12. The mutated replicative form DNA was transformed into E. coli strain JM109 and single plaques were picked and sequenced for verification of the mutation. The FGF mutated gene was then cut out of M13, ligated into the expression vector pFC80, which had the non-mutated form of the gene removed, and transformed into E. coli strain JM109. Single colonies were picked and the plasmids s quenced to verify that the mutation was present. Plasmids with correct

mutation were then transformed into the E. coli strain FICE 2 and single colonies from these transformations were used to obtain the mutant basic FGFs. An excellent level of expression, approximately 20 mg per liter of fermentation broth, was achieved.

2. Purification of mutagenized FGF

Cells were grown overnight in 20 ml of LB broth containing 100 μg/ml ampicillin. The next morning the cells were pelleted and transferred to 500 ml of M9 medium with 100 μg/ml ampicillin and grown for 7 hours. The cells were pelleted and resuspended in lysis solution (10 mM TRIS, pH 7.4, 150 mM NaCl, lysozyme, 10 μg/mL, aprotinin, 10 μg/mL, leupeptin, 10 μg/mL, pepstatin A, 10 μg/mL and 1 mM PMSF; 45-60 ml per 16 g of pellet) and incubated while stirring for 1 hour at room temperature. The solution was frozen and thawed three times and sonicated for 2.5 minutes. The suspension was centrifuged; the supernatant saved and the pellet resuspended in another volume of lysis solution without lysozyme, centrifuged again and the supernatants pooled. Extract volumes (40 ml) were diluted to 50 ml with 10 mM TRIS, pH 7.4 (buffer A). Pools were loaded onto a 5 ml Hi-Trap heparin-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated in 150 mM sodium chloride in buffer A. The column was washed with 0.6 M sodium chloride and 1 M sodium chloride in buffer A and then eluted with 2 M sodium chloride in buffer A. Peak fractions of the 2 M elution, as determined by optical density at 280 nm, were pooled and purity determined by gel electrophoresis. Yields were 10.5 mg of purified protein for the Cys^{78} mutant and 10.9 mg for the Cys^{96} mutant.

The biological activity of [C78S]FGF and [C96S]FGF was measured on adrenal capillary endothelial cells in culture. Cells were plated 3,000 per well of a 24 well plate in 1 ml of 10% calf serum-HDMEM. When cells were attached, samples were added in triplicate at the indicated concentration and incubated for 48 h at 37°C. An equal quantity of samples was added and further incubated for 48 h. Medium was aspirated; cells were treated with trypsin (1 ml volume) to remove cells to 9 ml of

Hematall diluent and counted in a Coulter Counter. The results show that the two mutants that retain virtually complete proliferative activity of native basic FGF as judged by the ability to stimulate endothelial cell proliferation in culture.

EXAMPLE 4

PREPARATION OF SAPORIN: DERIVATIZATION AND PURIFICATION OF MONO-DERIVATIZED SAPORIN

Saporin (SAP; 49 mg) at a concentration of 4.1 mg/ml was dialyzed against 0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.5. A 1.1 molar excess (563 μg in 156 μl of anhydrous ethanol) of SPDP (Pharmacia, Uppsala, Sweden) was added and the reaction mixture immediately agitated and put on a rocker platform for 30 minutes. The solution was then dialyzed against the same buffer. An aliquot of the dialyzed solution was examined for extent of derivatization according to the Pharmacia instruction sheet. The extent of derivatization was 0.86 moles of SPDP per mole of SAP. During these experiments, another batch of SAP was derivatized using an equimolar quantity of SPDP in the reaction mixture with a resulting 0.79 molar ratio of SPDP to SAP.

Derivatized SAP (32.3 mg) was dialyzed in 0.1 M sodium borate, pH 9.0 and applied to a Mono S 16/10 column equilibrated with 25 mM sodium chloride in dialysis buffer. A gradient of 25 mM to 125 mM sodium chloride in dialysis buffer was run to elute SAP and derivatized SAP. The flow rate was 4.0 ml/min. and 4 ml fractions were collected. Aliquots of fractions were assayed for protein concentration (BCA Protein Assay, Pierce Chemical, Chicago, IL) and for pyridylthione released by reducing agent. Individual fractions from 25 to 37 were analyzed for protein concentration and pyridyl-disulfide concentration and are presented in Table 5. Fractions 24-28 correspond to approximately 2 moles of 2-pyridyl disulfide per mole of SAP, 29-33 corresponds to one mole per mole and 34-37 contain non-derivatized SAP. These data indicate a separation according to the level of derivatization by SPDP. The initial eluting peak is composed of SAP

that is approximately di-derivatized; the second peak is mono-derivatized and the third peak shows no derivatization. The di-derivatized material accounts for 20% of the three peaks; the second accounts for 48% and the third peak contains 32%. Material from the second peak was pooled and gave an average ratio of pyridyl-disulfide to SAP of 0.95. Fraction 33 showed a divergent ratio of pyridine-2-thione to protein, perhaps because of its low concentration. It was excluded from the pool. The pooled material was used for the conjugation described here. Fractions that showed a ratio of SPDP to SAP greater than 0.85 but less than 1.05 were pooled, dialyzed against 0.1 M sodium chloride, 0.1 M sodium phosphate, pH 7.5 and used for derivatization with basic FGF. A pool of these materials had a molar ratio SPDP:SAP of 0.9 with a final yield of 4.6 mg.

TABLE 5

LEVELS OF DERIVATIZATION BY SPDP OF FRACTIONS FROM CHROMATOGRAPHY OF DERIVATIZED SAP

Fraction Number	Protein Concentration (μM)	Pyridine-2-Dithione Concentration (μM)	Pyridine-2-Thione/Protein Ratio
25	5.8	9.6	1.7
26	13.5	19.4	1.4
27	9.8	17.3	1.8
28	8.6	14.7	1.7
29	10.7	12.2	1.1
30	22.0	21.0	0.95
31	27.0	25.0	0.93
32	17.8	15.8	0.89
33	4.5	7.4	1.6
34	33.2	0	0
35	29.2	0	0
36	28.3	0	0
37	10.1	0	0

EXAMPLE 5

PREPARATION OF SAPORIN: PREPARATION OF MODIFIED SAPORIN

Instead of derivatizing SAP, SAP was modified by addition of a cysteine residueat the N-terminus-encoding portion of the DNA or the addition of a cysteine at position 4 or 10. The resulting saporin is then reacted with an available cysteine on an FGF to produce conjugates that are linked via the added Cys or Met-Cys on saporin.

Modified SAP has been prepared by modifying DNA encoding the saporin by inserting DNA encoding Met-Cys or Cys at position -1 or by replacing the Ile or the Asp codon within 10 or fewer residues of the N-terminus. The resulting DNA has been inserted into pET11a and pET15b and expressed in BL21 cells. The resulting saporin proteins are designated FPS1 (saporin with Cys at -1), FPS2 (saporin with Cys at position 4) and FPS3 (saporin with Cys at position 10). A plasmid that encodes FPS1 and that has been for expression of FPS1 has been designated PZ50B. Plasmids that encode FPS2 and that have been used for expression of FPS2 have been designated PZ51B (pET11a-based plasmid) and PZ51E (pet15b-based plasmid). Plasmids that encode FPS3 and that have been used for expression of FPS3 have been designated PZ52B (pET11a-based plasmid) and PZ52E (pet15b-based plasmid).

A. Materials and Methods

1. Bacterial strains

Novablue (NOVAGEN, Madison, WI) and BL21(DE3) (NOVAGEN, Madison WI).

2. DNA manipulations

DNA manipulations were performed as described in Examples 1 and 2.

Plasmid PZ1B (designated PZ1B1) described in Example 2 was used as the DNA template.

B. Preparation of saporin with an added cysteine residue at the N-terminus

1. Primers

(a) Primer #1 corresponding to the sense strand of saporin, nucleotides 472-492 of SEQ ID NO. 12, incorporates a NdeI site and adds a cys codon 5' to the first codon of the mature protein (between Met and Val):

CATATGTGTGTCACATCAATCACATTAGAT (SEQ ID NO. 34)

(b) Primer #2 - Antisense primer complements the coding sequence of saporin spanning nucleotides 547-567 of SEQ ID NO. 12 and contains a BamHI site:

CAGGTTTGGATCCTTTACGTT (SEQ ID NO. 35)

2. Isolation of saporin-encoding DNA

PZIB1 DNA was amplified by PCR as follows using the above primers. PZ1B DNA (1 μl) was mixed in a final volume of 100 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 μg of each primer. Next, 2.5 U TaqI DNA polymerase (Boehringer Mannheim) was added and the mixture was overlaid with 30 μl of mineral oil (Sigma). Incubations were done in a DNA Thermal Cycler (Ericomp). One cycle included a denaturation step (94°C for 1 min.), an annealing step (60°C for 2 min.), and an elongation step (72°C for 3 min.). After 35 cycles, a 10 μl aliquot of each reaction was run on a 1.5% agarose gel to verify the correct structure of the amplified product.

The amplified DNA was gel purified and digested with NdeI and BamHI and subcloned into NdeI and BamHI-digested pZ1B1. This digestion and subcloning step removed the FGF-encoding DNA and 5' portion of SAP up to the BamHI site at nucleotides 555-560 (SEQ ID No. 12) and replaced this portion with DNA encoding a saporin molecule that contains a cysteine residue at position -1 relative to the start site of the native mature SAP protein. The resulting plasmid is designated pZ50B1.

C. Preparation of saporin with a cysteine residue at position 4 or 10 of the native protein

These constructs were designed to introduce a cysteine residue at position 4 or 10 of the native protein by replacing the isoleucine residue at position 4 or the asparagine residue at position 10 with cysteine.

1. Materials

(a) Bacterial strains

The bacterial strains were Novablue and BL21(DE3) (NOVAGEN, Madison, WI).

(b) DNA manipulations

DNA manipulations as described above.

2. Preparation of modified SAP-encoding DNA

SAP was amplified by polymerase chain reaction (PCR) from the parental plasmid pZ1B1 encoding the FGF-SAP fusion protein.

(a) Primers

(1) The primer corresponding to the sense strand of saporin, spanning nucleotides 466-501 of SEQ ID NO. 12, incorporates a NdeI site and replaces the Ile codon with a Cys codon at position 4 of the mature protein (SEQ ID NO. 38):

CATATGGTCACATCATGTACATTAGATCTAGTAAAT.

(2) The primer corresponding to the sense strand of saporin, nucleotides 466-515 of SEQ ID NO. 12, incorporates a NdeI site and replaces the Asp codon with a cys codon at position 10 of the mature protein (SEQ ID NO. 39)

CATATGGTCACATCAATCACATTAGATCTAGTATGTCCGACCGCGGGTCA

(3) Primer #2 - Antisense primer complements the coding sequence of saporin spanning nucleotides 547-567 of SEQ ID NO. 12 and contains a BamHI site (SEQ ID NO. 35):

CAGGTTTGGATCCTTTACGTT.

(b) Amplification

The PCR reactions were performed as described above, using the following cycles: denaturation step 94°C for 1 min, annealing for 2 min at 60°C, and extension for 2 min at 72°C for 35 cycles. The amplified DNA was gel purified, digested with NdeI and BamHI, and subcloned into **NdeI** and **BamHI** digested pZ1B1. This digestion removed the FGF and 5' portion of SAP (up to the newly added BamHI) from the parental FGF-SAP vector (pZ1B1) and replaced this portion with a SAP molecule containing a CYS at position 4 or 10 relative to the start site of the native mature SAP protein. The resulting plasmids are designated pZ51B1 and pZ52B1, respectively.

D. **Cloning of DNA encoding SAP mutants in vector pET15b**

The initial step in this construction was the mutagenesis of the internal BamHI site at nucleotides 555-560 (SEQ ID NO. 12) in pZ1B1 by PCR using a sense primer corresponding to nucleotides 543-570 (SEQ ID NO. 12) but changing the G at nucleotide 555 (the third position in the Lys codon) to an A. The complement of the sense primer was used as the antisense primer. The PCR reactions were conducted as in B above. One μl of the resulting PCR product was used in a second PCR reaction using the same sense oligonucleotide as in B., above, in order to introduce a NdeI site and a Cys codon onto the 5' end of the saporin-encoding DNA. The antisense primer was complementary to the 3' end of the saporin protein and encoded a BamHI site for cloning and a stop codon (SEQ ID NO. 37):

GGATCCGCCTCGTTTGACTACTT.

The resulting plasmid was digested with NdeI/BamHI and inserted into pET15b (NOVAGEN, Madison, WI), which has a His-Tag™ leader sequence (SEQ ID NO. 36), that had also been digested NdeI/BamHI.

The SAP-Cys-4 and Sap-Cys-10 mutants were similarly inserted into pET15b using SEQ ID Nos. 38 and 39, respectively as the sense primers and SEQ ID NO. 37 as the antisense primer.

DNA encoding unmodified SAP (EXAMPLE 1) can be similarly inserted into a pet15b or pet11A and expressed as described below for the modified SAP-encoding DNA.

E. Expression of the modified saporin-encoding DNA

BL21(DE3) cells were transformed with the resulting plasmids and cultured as described in Example 2, except that all incubations were conducted at 30° C instead of 37° C. Briefly, a single colony was grown in LB AMP_{100} to and OD_{600} of 1.0-1.5 and then induced with IPTG (final concentration 0.1mM) for 2 h. The bacteria were spun down.

F. Purification of modified saporin

Lysis buffer (20 mM $NaPO_4$, pH 7.0, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin, 0.7 μg/ml pepstatin) was added to the rSAP cell paste (produced from pZ50B1 in BL21 cells, as described above) in a ratio of 1.5 ml buffer/g cells. This mixture was evenly suspended via a Polytron homogenizer and passed through a microfluidizer twice.

The resulting lysate was centrifuged 50,000 rpm for 45 min. The supernatant was diluted with SP Buffer A (20 mM $NaPO_4$, 1 mM EDTA, pH 7.0) so that the conductivity was below 2.5 mS/cm. The diluted lysate supernatant was then loaded onto a SP-Sepharose column, and a linear gradient of 0 to 30% SP Buffer B (1 M NaCl, 20 mM $NaPO_4$, 1 mM EDTA, pH 7.0) in SP Buffer A with a total of 6 column volumes was applied. Fractions containing rSAP were combined and the resulting rSAP had a purity of greater than 90%.

A buffer exchange step was used here to get the SP eluate into a buffer containing 50 mM $NaBO_3$, 1 mM EDTA, pH 8.5 (S Buffer A). This sample was then applied to a Resource S column (Pharmacia, Sweden) pre-equilibrated with S Buffer A. Pure rSAP was eluted off the column by 10 column volumes of a linear gradient of 0 to 300 mM NaCl in SP Buffer A. The final rSAP was approximately 98% pure and the overall yield of rSAP

was about 50% (the amount of rSAP in crude lysate was determined by ELISA).

In this preparation, ultracentrifugation was used to clarify the lysate; other methods, such as filtration and using floculents also can be used. In addition, Streamline S (PHARMACIA, Sweden) may also be used for large scale preparations.

EXAMPLE 6

A. Cytotoxicity assays of conjugates

Cytotoxicity experiments were performed with the Promega (Madison, WI) CellTiter 96 Cell Proliferation/Cytotoxicity Assay. Cell types used were SK-Mel-28, human melanoma Swiss 3T3 mouse fibroblasts (from Dr. Pamela Maher, La Jolla, CA), B16F10, mouse melanoma, PA-1, human ovarian carcinoma (from Dr. Julie Beitz, Roger Williams Hospital, Providence RI), and baby hamster kidney (BHK) [obtained from the American Type Culture Collection (ATCC)]. 2500 cells were plated per well.

B. Coupling of FGF muteins to SAP

1. Chemical Synthesis of [C78S]FGF-SAP (CCFS2) and [C96S]FGF-SAP (CCFS3)

[C78S]FGF or [C96S]FGF (1 mg; 56 nmol) that had been dialyzed against phosphate-buffered saline was added to 2.5 mg mono-derivatized SAP (a 1.5 molar excess over the basic FGF mutants) and left on a rocker platform overnight. The next morning the ultraviolet-visible wavelength spectrum was taken to determine the extent of reaction by the release of pyridylthione, which adsorbs at 343 nm with a known extinction coefficient. The ratio of pyridylthione to basic FGF mutant for [C78S]FGF was 1.05 and for [C96S]FGF was 0.92. The reaction mixtures were treated identically for purification in the following manner: reaction mixture was passed over a HiTrap heparin-Sepharose column (1 ml) equilibrated with 0.15 M sodium chloride in buffer A at a flow rate of 0.5 ml/min. The column was washed with 0.6 M NaCl and 1.0 M NaCl in buffer A and the

product eluted with 2.0 M NaCl in buffer A. Fractions (0.5 ml) were analyzed by gel electrophoresis and absorbance at 280 nm. Peak tubes were pooled and dialyzed versus 10 mM sodium phosphate, pH 7.5 and applied to a Mono-S 5/5 column equilibrated with the same buffer. A 10 ml gradient between 0 and 1.0 M sodium chloride in equilibration buffer was used to elute the product. Purity was determined by gel electrophoresis and peak fractions were pooled. The yield for [C78S]FGF-SAP was 1.6 mg (60% with respect to starting amount of [C78S]FGF) and was 0.96 mg [C96S]FGF-SAP (35%).

Virtually 100% of the mutant FGFs reacted with mono-derivatized SAP ([C78S]FGF: 105%, [C96S]FGF: 92%). Because the free surface cysteine of each mutant acts as a free sulfhydryl, it was unnecessary to reduce cysteines after purification from the bacteria. The resulting product was purified by heparin-Sepharose (data not shown), thus establishing that heparin binding activity of the conjugate is retained.

Coomassie staining and Western blotting of the purified proteins showed a prominent band at a molecular weight of about 48,000, corresponding to the combined molecular weights of SAP and bFGF. A much lighter band at a slightly lower molecular weight was detected and attributed to the described mobility of an artifact produced by the high isoelectric point (10.5) (Gelfi et al. (1987) J. Biochem. Biophys. Meth. 15:41-48) of SAP that causes a smearing in SDS gel electrophoresis (see, e.g., Lappi et al. (1985) Biochem. Biophys. Res. Commun. 129:934-942). No higher molecular weight bands, corresponding to conjugates containing more than one molecule of SAP per molecule of basic FGF or more than one molecule of basic FGF per molecule of SAP were detected on Coomassie-stained gels of [C78S]FGF-SAP) and of ([C96S]FGF-SAP). Such bands were present in lanes on the gel in which an equal quantity (by weight) of heterogeneous FGF-SAP, synthesized from wild-type bFGF and non-purified derivatized SAP, had been loaded.

Western blotting using antibodies to SAP or basic FGF revealed that, while 480 ng of either [C78S]FGF-SAP or [C96S]FGF-SAP results in a well-visualized band (with the additional slight lower molecular weight band) the same quantity of conjugate produced by the previous procedure is almost undetectable. As in the Coomassie staining, the Western blotting of the mutant FGF-SAPs reveals much greater homogeneity than with heterogeneous FGF-SAP synthesized with non-mutagenized basic FGF and non-purified derivatized SAP.

2. Preparation of [C96S]FGF-rSAP (CCFS4)

Recombinant saporin that has the cys added at the N-terminus (SAP-CYS-(-1)) that was cloned and expressed in BL21 cells and isolated as described in EXAMPLE 4 was coupled to [C96S]FGF using (5,5'-dithiobis-(2-nitrobenzoic acid)) DTNB also called Ellman's reagent. The rSAP and [C96S]FGF were each treated with 10 mM dithiothreitol (DTT), incubated for 1 h at room temperature, and the DTT was removed by gel filtration in conjugation buffer (0.1 M $NaPO_4$, 100 NaCl and 1 mM EDTA, pH 7.5). A 100-fold molar excess of DTNB was added to the rSAP, incubated for 1 h at room temperature. Unreacted DTNB was removed by gel filtration. The [C96S]FGF was added to DTNB-treated SAP (3:1 molar ratio of [C96S]FGF:SAP) and incubated at room temperature for about 1 hr or for 16 hrs at 4° C. The mixture was loaded on heparin sepharose in 10 mM $NaPO_4$, 1 mM EDTA, pH 6 and the conjugate and free [C96S]FGF were eluted with 2 M NaCl in 10 mM $NaPO_4$, 1 mM EDTA, pH 6. The free [C96S]FGF was removed by gel filtration on Sephacryl S100 (Pharmacia). The resulting conjugate was designated CCFS4.

C. Cytotoxicity of [C78S]FGF-SAP (CCFS2), [C96S]FGF-SAP (CCFS3) and [C96S]FGF-rSAP (CCFS4)

Cytotoxicity of the two mutant FGF-SAPs to several cell types has been tested. Heterogeneous FGF-SAP (CCFS1) is very cytotoxic to SK-MEL-28 cells, human melanoma cells, with an ED_{50} of approximately 8 ng/ml. The mutant FGF-SAPs are also potently cytotoxic to these cells.

[C78S]FGF-SAP and [C96S]FGF-SAP each have an ED_{50} comparable to the heterogeneous chemically conjugates, indicting that mutant FGFs are able to internalize SAP to virtually the same extent as the heterogeneous FGF-SAP.

Similar results were obtained with an ovarian carcinoma cell type, PA-1, Swiss 3T3 cells, B16F10, a mouse melanoma and BHK cells (Table 6).

CCFS4 was tested in the in vitro cytotoxicity assay and its activity is at least as good to the wild-type chemical conjugate (CCFS1).

TABLE 6

CYTOTOXICITY OF HOMOGENEOUS AND HETEROGENOUS FGF-SAPs TO CELL LINES

Cell Type	ED_{50}s (ng/ml)		Heterogeneous FGF-SAP
	[C96S]FGF-SAP	[C78S]FGF-SAP	
SK-MEL-28	8	12	8
Swiss 3T3	60	100	40
PA-1	70	100	40
B16F10	2	3	2
BHK	20	25	15

D. Preparation of homogeneous mixtures of FGF-SAP muteins by splicing by overlap extension (SOE)

1. Conversion of Cys 78 to Ser 78

(a) Materials

(1) Plasmids

Plasmid PZ1B (designated PZ1B1) described in Example 2 was used as the DNA template. The primers were prepared as follows:

(2) Primers

(a) Primer #1 spanning the NdeI site at the 5' end of the FGF-encoding DNA from plasmid pZIB:

AAATACTTACATATGGCAGCAGGATC (SEQ ID NO. 18).

(b) Primer #2 - Antisense primer to nucleotides spanning the Cys 78 (nucleotides 220-249 of SEQ ID NO. 12 with base change to generate Ser 78):

CAGGTAACGGTTAGCAGACACTCCTTTGAT (SEQ ID NO. 19).

(c) Primer #3 - Sense primer to nucleotides spanning the Cys 78 (nucleotides 220-249 of SEQ ID NO. 12 with base change to generate Ser 78):

ATCAAAGGAGTGTCTGCTAACCGTTACCTG (SEQ ID NO. 20).

(d) Primer #4 - Antisense primer to spanning the NcoI site of FGF in pZ1B (corresponding to nucleotides 456-485 of SEQ ID NO. 12):

GTGATTGATGTGACCATGGCGCTCTTAGCA (SEQ ID NO. 21).

(b) Reactions

(1) Reaction A

PZ1B1 DNA (100 ng) was mixed (final volume of 100 μl upon addition of the Taq polymerase) with primer #1 (50 μM); primer #2 (50 μM), 10 mM Tri-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 2 mM $MgCl_2$, 0.2 mM dNTPs.

(2) Reaction B

Same as above except that primer #3 (50 μM) and primer #4 (50 μM) were used in place of primers #1 and #2.

Each reaction mixture was heated to 95° C for 5 min, 0.5 U TaqI DNA polymerase (1 μl; Boehringer Mannheim) was added and the mixture was overlaid with 100 μl of mineral oil (Perkin Elmer Cetus). Incubations were done in a DNA Thermal Cycler (Ericomp). Each cycle included a

denaturation step (95°C for 1 min.), an annealing step (60°C for 1.5 min.), and an elongation step (75°C for 3 min.). After 20 cycles, the reaction mixture was incubated at 75° C for 10 minutes for a final elongation. The products were resolved on a 2% agarose gel and DNA of the correct size (247 bp and 250 bp) was purified. The ends were repaired by adding nucleoside triphosphates and Klenow DNA polymerase.

(3) Reaction C

One μl of each product of reactions A and B were mixed (final volume of 100 μL upon addition of Taq polymerase) with primers #1 and #4 (final concentration of each was 50 μM); 10 mM Tri-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 2 mM $MgCl_2$, 0.2 mM dNTPs.

The resulting reaction mixture was heated to 95° C for 5 min, 0.5 U TaqI DNA polymerase (1 μl; Boehringer Mannheim) was added and the mixture was overlaid with 100 μl of mineral oil (Perkin Elmer Cetus). Incubations were done in a DNA Thermal Cycler (Erricomp). Each cycle included a denaturation step (95°C for 1 min.), an annealing step (60°C for 1.5 min.), and an elongation step (75°C for 3 min.), followed, after 20 cycles, by a final elongation step at 75° C for 10 minutes.

The amplified product was resolved on a 1.5% agarose gel and the correct size fragment (460 bp), designated FGFC78S-SAP was purified.

2. Generation of DNA encoding FGFC78/C96S-SAP

(a) Materials

(1) Template

DNA encoding FGFC78S-SAP.

(2) Primers

(a) Primer #5-Sense primer spanning the Cys 96 (nucleotides 275-300 of SEQ ID NO. 12 with base change to generate Ser 96)

TGGCTTCTAAATCTGTTACGGATGAG (SEQ ID NO. 22).

(b) **Primer #6-Antisense primer spanning the Cys 96 (nucleotides 275-300 of SEQ ID NO. 12 with base change to generate Ser 96):**

CTCATCCGTAACAGATTTAGAAGCCA (SEQ ID NO. 23).

(b) Reactions

(1) Reaction D

FGFC78S-SAP-encoding DNA (100 ng) was mixed (final volume of 100 μl upon addition of the Taq polymerase) with primer #1 (50 μM); primer #5 (50 μM), 10 mM Tri-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 2 mM $MgCl_2$ and 0.2 mM dNTPs.

(2) Reaction E

Same as above, except that primers #4 and #6 (50 μM final concentration of each) were used instead of primers #1 and #5.

Each reaction mixture was heated to 95° C for 5 min, 0.5 U TaqI DNA polymerase (1 μl;Boehringer Mannheim) was added and the mixture was overlaid with 100 μl of mineral oil (Perkin Elmer Cetus). Incubations were done in a DNA Thermal Cycler (Ericomp). Each cycle included a denaturation step (95°C for 1 min.), an annealing step (60°C for 1.5 min.), and an elongation step (75°C for 3 min.) for 20 cycles, followed by a final elongation step at 75° C for 10 minutes. The products were resolved on a 2% agarose gel and DNA of the correct size (297 bp and 190 bp) was purified. The ends were repaired by adding nucleoside triphosphates and Klenow DNA polymerase.

(3) Reaction F

The product of reactions D and E (100 ng of each) were mixed (final volume of 100 μL upon addition of Taq polymerase) with primers #1 and #4 and amplified as described above. The amplified product resolved on a 1.5% agarose gel and the correct size fragment (465 bp) was purified. The resulting product, DNA that encodes FGFC78/96S-SAP, had NdeI and NcoI ends. It was digested with NdeI and NcoI and ligated into NdeI/NcoI-digested PZ1B1 and into NdeI/NcoI-digested PZ1C1 (PZIC described in

Example 2 above). The resulting constructs were designated PZ2B1 and PZ2C1, respectively.

E. Expression of the recombinant FGFC78/96S-SAP fusion proteins (FPFS4) from PZ2B1 and PZ2C1

The two-stage method described above for production of FPFS1 was used to produce recombinant FGFC78/96S-SAP protein (hereinafter FPFS4).

Two hundred and fifty mls. of LB medium containing ampicillin (100 μg/ml) were inoculated with a fresh glycerol stock of PZ1B. Cells were grown at 30° C in an incubator shaker to an OD_{600} of 0.7 and stored overnight at 4° C. The following day the cells were pelleted and resuspended in fresh LB medium (no ampicillin). The cells were divided into 5 1-liter batches and grown at 30° C in an incubator shaker to an OD_{600} of 1.5. IPTG (SIGMA CHEMICAL, St. Louis, MO) was added to a final concentration of 0.1 mM and growth was continued for about 2 to 2.5 hours at which time cells were harvested by centrifugation.

In order to grow PZ2C1, prior to induction, the cells were grown in medium containing kanamycin (50μg/ml) in place of ampicillin.

F. Biological Activity

The cytotoxicity of the mutein FGF-SAP produced from PZ2B1 (FPFS4) was assessed on SK MEL 28 cells and was at least equivalent to the activity of the wild type FGF-SAP chemical conjugate, and recombinant FGF-SAP produced from PZ1B1.

The in vivo activity of the mutein FGF-SAP produced from PZ2B1 has been tested in animals, and it appears to be less toxic than FGF-SAP from PZ1B1 (FPFS1).

EXAMPLE 7

THERAPEUTIC ACTIVITY OF THE WILD-TYPE CHEMICAL CONJUGATE AND FUSION PROTEIN bFGF-SAP IN THE MOUSE TUMOR XENOGRAFT MODEL

A. Materials and methods

The methods set forth below were performed substantially as described in Beitz et al. (1992) Cancer Research 52:227-230).

(1) Study Design

Sixty-three athymic mice bearing subcutaneous tumors received four weekly bolus IV injections of the test materials. Tumor volumes were measured twice weekly for 61 days.

(2) Test Materials

Wild-type chemical conjugate bFGF-SAP was supplied in Dulbecco's phosphate buffered saline (PBS) at a concentration of 1.0 mg/ml. Fusion protein bFGF-SAP in E. coli was supplied in Dulbecco's PBS at a concentration of 9.0 mg/ml. Basic FGF was supplied in Dulbecco's PBS at a concentration of 1.0 mg/ml. Saporin was supplied in Dulbecco's PBS (0.01 M Phosphate, 0.14 M NaCl, pH 7.4) at a concentration of 1.0 mg/ml. All dilutions were made in Dulbecco's PBS with 0.1% bovine serum albumin (NB 1005-18).

(3) Species

Female Balb/c nu/nu athymic mice (Roger Williams Hospital Animal Facility, Providence, RI), 8-12 weeks old, were maintained in an aseptic environment. Sixty-three animals were selected for the study, and body weights ranged from 25-30 grams the day prior to dosing.

(4) Husbandry

Animals were maintained in a quarantined room and handled under aseptic conditions. Food and water were supplied ad libitum throughout the experiment.

(5) Tumor Cells

PA-1 human ovarian teratocarcinoma cells were obtained from the American Type Culture Collection (Rockville, MD; ATCC accession no. CRL1572) were grown in modified Eagle's medium supplemented with 10% fetal calf serum.

(6) Tumor Implantation

Five days prior to injection of the test material, mice received a subcutaneous injection of tumor cells (approximately 2×10^6 PA-1 human ovarian teratocarcinoma cells/mouse) in the right rear flank.

(7) Tumor Size Measurements

Calipers were used to measure the dimensions of each tumor. Measurements (mm) of maximum and minimum width were performed prior to injection of the test material and at bi-weekly intervals for 61 days. Tumor volumes (mm^3) were computed using the formula Volume = [(minimum measurement)^2(maximum measurement)]/2.

(8) Dose Preparation

Dosing material was prepared by mixing the test material with appropriate volumes of PBS/0.1% BSA to achieve the final doses.

(9) Dosing Procedures

Individual syringes were prepared for each animal. Mice received four weekly IV injections (250-300 ul) into the tail vein on days 5, 12, 19 and 26 with day 1 designated as the day that the tumor cells were injected into the mice. Doses were individualized for differences in body weight.

B. Results - Inhibition of tumor growth

In all animals, tumors were measured prior to injection of the test material and at bi-weekly intervals for 61 days. Tumors from animals in all groups were approximately 55-60 mm^3 on day 5 when treatment began. The vehicle-treated group (PBS with 0.1% BSA) showed a 50-fold increase in tumor volume over the 61 days of the study. The other control groups demonstrated similar levels of tumor growth: the SAP control group showed a 30-fold increase, the bFGF control group showed a 50-fold increase, and the bFGF plus SAP group showed a 50-fold increase in tumor volume. In all the control groups, the rate of growth of the tumor was fairly consistent over the 61-day period. In the treated groups, with wild-type chemical conjugate bFGF-SAP and fusion protein bFGF-SAP , there appeared to be a statistically significant dose-related suppression in tumor growth compared to controls over the first 30 days; however, tumor volumes increased again after this period such that there was no longer a statistical diff rence between the treated and control groups.

The 50 μg/kg/week fusion protein bFGF-SAP-treated groups exhibited tumor volumes that were 29% of controls, but a statistical comparison to controls was not done because only two animals in the treated group survived to 30 days. The fusion protein bFGF-SAP 5.0 μg/kg/week dose achieved significant suppression of tumor growth, with tumor volumes at 48% of control values. The 0.5 μg/kg/week fusion protein bFGF-SAP group showed significant suppression of tumor growth to day 26 when tumors were at 71% of controls. There was no statistical difference between tumor volumes in the 0.5 μg/kg/week wild-type chemical conjugate bFGF-SAP and fusion protein bFGF-SAP groups at 30 days. A statistical comparison of the two 50 μg/kg/week treatment groups was not done because there were only two surviving animals in the fusion protein bFGF-SAP group.

All seven animals survived the 61-day study in all groups with the exception of the 50 μg/kg/week chemical conjugate bFGF-SAP group (3 of 7 survived to 61 days) and the 50 μg/kg/week fusion protein bFGF-SAP group (1 of 7 survived to 61 days).

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Prizm Pharmaceuticals
(B) STREET: 10655 Sorrento Valley Road, Suite 200
(C) CITY: San Diego
(D) STATE: California
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 92121

(i) APPLICANT:
(A) NAME: The Whittier Institute for Diabetes and Endocrinology
(B) STREET: 9894 Genesee Avenue
(C) CITY: La Jolla
(D) STATE: California
(D) COUNTRY: USA
(E) POSTAL CODE (ZIP): 92037

(ii) TITLE OF INVENTION: MONOGENOUS PREPARATIONS OF CYTOTOXIC CONJUGATES

(iii) NUMBER OF SEQUENCES: 39

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/145,829
(B) FILING DATE: 29-OCT-1993

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/099,924
(B) FILING DATE: 02-AUG-1993

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

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(A) NAME/KEY: misc_recomb
(B) LOCATION: 6..11
(D) OTHER INFORMATION: /standard_name= "EcoRI Restriction Site"

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 12..30
(D) OTHER INFORMATION: /function= "N-terminal extension"
/product= "Native saporin signal peptide"

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(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 6..11
(D) OTHER INFORMATION: /standard_name= "EcoRI Restriction Site"

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(A) NAME/KEY: terminator
(B) LOCATION: 23..25
(D) OTHER INFORMATION: /note= "Anti-sense stop codon"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 26..30
(D) OTHER INFORMATION: /note= "Anti-sense to carboxyl terminus of mature peptide"

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 804 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..804

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..804
(D) OTHER INFORMATION: /note= "Nucleotide sequence corresponding to the clone M13 mp18-G4 in Example I.B.2."

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 46..804
(D) OTHER INFORMATION: /product= ""Saporin""

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Lys Asp Tyr Asp Phe Gly Phe Gly Lys Val Arg Gln Val Lys Asp Leu
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(A) LENGTH: 804 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..804

(ix) FEATURE:
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(B) LOCATION: 1..804
(D) OTHER INFORMATION: /note= "Nucleotide sequence corresponding to the clone M13 mp18-G1 in Example I.B.2."

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 46..804
(D) OTHER INFORMATION: /product= "Saporin"

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TAC GGT GGT ACC GAC ATA GCC GTG ATA GGC CCA CCT TCT AAA GAA AAA       192
Tyr Gly Gly Thr Asp Ile Ala Val Ile Gly Pro Pro Ser Lys Glu Lys
     35                  40                  45

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Phe Leu Arg Ile Asn Phe Gln Ser Ser Arg Gly Thr Val Ser Leu Gly
 50                  55                  60                  65

CTA AAA CGC GAT AAC TTG TAT GTG GTC GCG TAT CTT GCA ATG GAT AAC       288
Leu Lys Arg Asp Asn Leu Tyr Val Val Ala Tyr Leu Ala Met Asp Asn
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ACG AAT GTT AAT CGG GCA TAT TAC TTC AGA TCA GAA ATT ACT TCC GCC        336
Thr Asn Val Asn Arg Ala Tyr Tyr Phe Arg Ser Glu Ile Thr Ser Ala
              85                  90                  95

GAG TTA ACC GCC CTT TTC CCA GAG GCC ACA ACT GCA AAT CAG AAA GCT        384
Glu Leu Thr Ala Leu Phe Pro Glu Ala Thr Thr Ala Asn Gln Lys Ala
      100                 105                 110

TTA GAA TAC ACA GAA GAT TAT CAG TCG ATC GAA AAG AAT GCC CAG ATA        432
Leu Glu Tyr Thr Glu Asp Tyr Gln Ser Ile Glu Lys Asn Ala Gln Ile
  115                 120                 125

ACA CAG GGA GAT AAA TCA AGA AAA GAA CTC GGG TTG GGG ATC GAC TTA        480
Thr Gln Gly Asp Lys Ser Arg Lys Glu Leu Gly Leu Gly Ile Asp Leu
130                 135                 140                 145

CTT TTG ACG TCC ATG GAA GCA GTG AAC AAG AAG GCA CGT GTG GTT AAA        528
Leu Leu Thr Ser Met Glu Ala Val Asn Lys Lys Ala Arg Val Val Lys
                150                 155                 160

AAC GAA GCT AGG TTT CTG CTT ATC GCT ATT CAA ATG ACA GCT GAG GTA        576
Asn Glu Ala Arg Phe Leu Leu Ile Ala Ile Gln Met Thr Ala Glu Val
            165                 170                 175

GCA CGA TTT CGG TAC ATT CAA AAC TTG GTA ACT AAG AAC TTC CCC AAC        624
Ala Arg Phe Arg Tyr Ile Gln Asn Leu Val Thr Lys Asn Phe Pro Asn
        180                 185                 190

AAG TTC GAC TCG GAT AAC AAG GTG ATT CAA TTT GAA GTC AGC TGG CGT        672
Lys Phe Asp Ser Asp Asn Lys Val Ile Gln Phe Glu Val Ser Trp Arg
    195                 200                 205

AAG ATT TCT ACG GCA ATA TAC GGA GAT GCC AAA AAC GGC GTG TTT AAT        720
Lys Ile Ser Thr Ala Ile Tyr Gly Asp Ala Lys Asn Gly Val Phe Asn
210                 215                 220                 225

AAA GAT TAT GAT TTC GGG TTT GGA AAA GTG AGG CAG GTG AAG GAC TTG        768
Lys Asp Tyr Asp Phe Gly Phe Gly Lys Val Arg Gln Val Lys Asp Leu
                230                 235                 240

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Gln Met Gly Leu Leu Met Tyr Leu Gly Lys Pro Lys
            245                 250
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..804

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..804
(D) OTHER INFORMATION: /note= "Nucleotide sequence

corresponding to the clone M13 mp18-G2 in Example I.B.2."

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 46..804
(D) OTHER INFORMATION: /product= "Saporin"

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-15                 -10                 -5                  1

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             5                  10                  15

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Ser Phe Val Asp Lys Ile Arg Asn Asn Val Lys Asp Pro Asn Leu Lys
         20                  25                  30

TAC GGT GGT ACC GAC ATA GCC GTG ATA GGC CCA CCT TCT AAA GAT AAA       192
Tyr Gly Gly Thr Asp Ile Ala Val Ile Gly Pro Pro Ser Lys Asp Lys
     35                  40                  45

TTC CTT AGA ATT AAT TTC CAA AGT TCC CGA GGA ACG GTC TCA CTT GGC       240
Phe Leu Arg Ile Asn Phe Gln Ser Ser Arg Gly Thr Val Ser Leu Gly
 50                  55                  60                  65

CTA AAA CGC GAT AAC TTG TAT GTG GTC GCG TAT CTT GCA ATG GAT AAC       288
Leu Lys Arg Asp Asn Leu Tyr Val Val Ala Tyr Leu Ala Met Asp Asn
                 70                  75                  80

ACG AAT GTT AAT CGG GCA TAT TAC TTC AAA TCA GAA ATT ACT TCC GCC       336
Thr Asn Val Asn Arg Ala Tyr Tyr Phe Lys Ser Glu Ile Thr Ser Ala
             85                  90                  95

GAG TTA ACC GCC CTT TTC CCA GAG GCC ACA ACT GCA AAT CAG AAA GCT       384
Glu Leu Thr Ala Leu Phe Pro Glu Ala Thr Thr Ala Asn Gln Lys Ala
        100                 105                 110

TTA GAA TAC ACA GAA GAT TAT CAG TCG ATC GAA AAG AAT GCC CAG ATA       432
Leu Glu Tyr Thr Glu Asp Tyr Gln Ser Ile Glu Lys Asn Ala Gln Ile
    115                 120                 125

ACA CAG GGA GAT AAA AGT AGA AAA GAA CTC GGG TTG GGG ATC GAC TTA       480
Thr Gln Gly Asp Lys Ser Arg Lys Glu Leu Gly Leu Gly Ile Asp Leu
130                 135                 140                 145

CTT TTG ACG TTC ATG GAA GCA GTG AAC AAG AAG GCA CGT GTG GTT AAA       528
Leu Leu Thr Phe Met Glu Ala Val Asn Lys Lys Ala Arg Val Val Lys
                150                 155                 160

AAC GAA GCT AGG TTT CTG CTT ATC GCT ATT CAA ATG ACA GCT GAG GTA       576
Asn Glu Ala Arg Phe Leu Leu Ile Ala Ile Gln Met Thr Ala Glu Val
            165                 170                 175

GCA CGA TTT AGG TAC ATT CAA AAC TTG GTA ACT AAG AAC TTC CCC AAC       624
Ala Arg Phe Arg Tyr Ile Gln Asn Leu Val Thr Lys Asn Phe Pro Asn
        180                 185                 190
```

```
AAG TTC GAC TCG GAT AAC AAG GTG ATT CAA TTT GAA GTC AGC TGG CGT        672
Lys Phe Asp Ser Asp Asn Lys Val Ile Gln Phe Glu Val Ser Trp Arg
    195                 200                 205

AAG ATT TCT ACG GCA ATA TAC GGG GAT GCC AAA AAC GGC GTG TTT AAT        720
Lys Ile Ser Thr Ala Ile Tyr Gly Asp Ala Lys Asn Gly Val Phe Asn
210                 215                 220                 225

AAA GAT TAT GAT TTC GGG TTT GGA AAA GTG AGG CAG GTG AAG GAC TTG        768
Lys Asp Tyr Asp Phe Gly Phe Gly Lys Val Arg Gln Val Lys Asp Leu
                230                 235                 240

CAA ATG GGA CTC CTT ATG TAT TTG GGC AAA CCA AAG                        804
Gln Met Gly Leu Leu Met Tyr Leu Gly Lys Pro Lys
            245                 250
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 804 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..804

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..804
(D) OTHER INFORMATION: /note= "Nucleotide sequence corresponding to the clone M13 mp18-G7 in Example I.B.2."

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 46..804
(D) OTHER INFORMATION: /product= "Saporin"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
GCA TGG ATC CTG CTT CAA TTT TCA GCT TGG ACA ACA ACT GAT GCG GTC         48
Ala Trp Ile Leu Leu Gln Phe Ser Ala Trp Thr Thr Thr Asp Ala Val
-15                 -10                 -5                  1

ACA TCA ATC ACA TTA GAT CTA GTA AAT CCG ACC GCG GGT CAA TAC TCA         96
Thr Ser Ile Thr Leu Asp Leu Val Asn Pro Thr Ala Gly Gln Tyr Ser
              5                  10                  15

TCT TTT GTG GAT AAA ATC CGA AAC AAC GTA AAG GAT CCA AAC CTG AAA        144
Ser Phe Val Asp Lys Ile Arg Asn Asn Val Lys Asp Pro Asn Leu Lys
          20                  25                  30

TAC GGT GGT ACC GAC ATA GCC GTG ATA GGC CCA CCT TCT AAA GAA AAA        192
Tyr Gly Gly Thr Asp Ile Ala Val Ile Gly Pro Pro Ser Lys Glu Lys
      35                  40                  45

TTC CTT AGA ATT AAT TTC CAA AGT TCC CGA GGA ACG GTC TCA CTT GGC        240
Phe Leu Arg Ile Asn Phe Gln Ser Ser Arg Gly Thr Val Ser Leu Gly
  50                  55                  60                  65
```

```
CTA AAA CGC GAT AAC TTG TAT GTG GTC GCG TAT CTT GCA ATG GAT AAC       288
Leu Lys Arg Asp Asn Leu Tyr Val Val Ala Tyr Leu Ala Met Asp Asn
                  70                  75                  80

ACG AAT GTT AAT CGG GCA TAT TAC TTC AGA TCA GAA ATT ACT TCC GCC       336
Thr Asn Val Asn Arg Ala Tyr Tyr Phe Arg Ser Glu Ile Thr Ser Ala
              85                  90                  95

GAG TTA ACC GCC CTT TTC CCA GAG GCC ACA ACT GCA AAT CAG AAA GCT       384
Glu Leu Thr Ala Leu Phe Pro Glu Ala Thr Thr Ala Asn Gln Lys Ala
         100                 105                 110

TTA GAA TAC ACA GAA GAT TAT CAG TCG ATC GAA AAG AAT GCC CAG ATA       432
Leu Glu Tyr Thr Glu Asp Tyr Gln Ser Ile Glu Lys Asn Ala Gln Ile
     115                 120                 125

ACA CAG GGA GAT AAA TCA AGA AAA GAA CTC GGG TTG GGG ATC GAC TTA       480
Thr Gln Gly Asp Lys Ser Arg Lys Glu Leu Gly Leu Gly Ile Asp Leu
130                 135                 140                 145

CTT TTG ACG TCC ATG GAA GCA GTG AAC AAG AAG GCA CGT GTG GTT AAA       528
Leu Leu Thr Ser Met Glu Ala Val Asn Lys Lys Ala Arg Val Val Lys
                  150                 155                 160

AAC GAA GCT AGA TTC CTT CTT ATC GCT ATT CAG ATG ACG GCT GAG GCA       576
Asn Glu Ala Arg Phe Leu Leu Ile Ala Ile Gln Met Thr Ala Glu Ala
              165                 170                 175

GCA CGA TTT AGG TAC ATA CAA AAC TTG GTA ATC AAG AAC TTT CCC AAC       624
Ala Arg Phe Arg Tyr Ile Gln Asn Leu Val Ile Lys Asn Phe Pro Asn
         180                 185                 190

AAG TTC AAC TCG GAA AAC AAA GTG ATT CAG TTT GAG GTT AAC TGG AAA       672
Lys Phe Asn Ser Glu Asn Lys Val Ile Gln Phe Glu Val Asn Trp Lys
     195                 200                 205

AAA ATT TCT ACG GCA ATA TAC GGG GAT GCC AAA AAC GGC GTG TTT AAT       720
Lys Ile Ser Thr Ala Ile Tyr Gly Asp Ala Lys Asn Gly Val Phe Asn
210                 215                 220                 225

AAA GAT TAT GAT TTC GGG TTT GGA AAA GTG AGG CAG GTG AAG GAC TTG       768
Lys Asp Tyr Asp Phe Gly Phe Gly Lys Val Arg Gln Val Lys Asp Leu
                  230                 235                 240

CAA ATG GGA CTC CTT ATG TAT TTG GGC AAA CCA AAG                       804
Gln Met Gly Leu Leu Met Tyr Leu Gly Lys Pro Lys
              245                 250
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 804 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..804

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..804
(D) OTHER INFORMATION: /note= "Nucleotide sequence corresponding to the clone M13 mp18-G9 in Example I.B.2."

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 46..804
(D) OTHER INFORMATION: /product= "Saporin"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
GCA TGG ATC CTG CTT CAA TTT TCA GCT TGG ACA ACA ACT GAT GCG GTC        48
Ala Trp Ile Leu Leu Gln Phe Ser Ala Trp Thr Thr Thr Asp Ala Val
-15                 -10                 -5                  1

ACA TCA ATC ACA TTA GAT CTA GTA AAT CCG ACC GCG GGT CAA TAC TCA        96
Thr Ser Ile Thr Leu Asp Leu Val Asn Pro Thr Ala Gly Gln Tyr Ser
             5                  10                  15

TCT TTT GTG GAT AAA ATC CGA AAC AAC GTA AAG GAT CCA AAC CTG AAA       144
Ser Phe Val Asp Lys Ile Arg Asn Asn Val Lys Asp Pro Asn Leu Lys
        20                  25                  30

TAC GGT GGT ACC GAC ATA GCC GTG ATA GGC CCA CCT TCT AAA GAA AAA       192
Tyr Gly Gly Thr Asp Ile Ala Val Ile Gly Pro Pro Ser Lys Glu Lys
    35                  40                  45

TTC CTT AGA ATT AAT TTC CAA AGT TCC CGA GGA ACG GTC TCA CTT GGC       240
Phe Leu Arg Ile Asn Phe Gln Ser Ser Arg Gly Thr Val Ser Leu Gly
50                  55                  60                  65

CTA AAA CGC GAT AAC TTG TAT GTG GTC GCG TAT CTT GCA ATG GAT AAC       288
Leu Lys Arg Asp Asn Leu Tyr Val Val Ala Tyr Leu Ala Met Asp Asn
                70                  75                  80

ACG AAT GTT AAT CGG GCA TAT TAC TTC AGA TCA GAA ATT ACT TCC GCC       336
Thr Asn Val Asn Arg Ala Tyr Tyr Phe Arg Ser Glu Ile Thr Ser Ala
            85                  90                  95

GAG TTA ACC GCC CTT TTC CCA GAG GCC ACA ACT GCA AAT CAG AAA GCT       384
Glu Leu Thr Ala Leu Phe Pro Glu Ala Thr Thr Ala Asn Gln Lys Ala
        100                 105                 110

TTA GAA TAC ACA GAA GAT TAT CAG TCG ATT GAA AAG AAT GCC CAG ATA       432
Leu Glu Tyr Thr Glu Asp Tyr Gln Ser Ile Glu Lys Asn Ala Gln Ile
    115                 120                 125

ACA CAA GGA GAT CAA AGT AGA AAA GAA CTC GGG TTG GGG ATT GAC TTA       480
Thr Gln Gly Asp Gln Ser Arg Lys Glu Leu Gly Leu Gly Ile Asp Leu
130                 135                 140                 145

CTT TCA ACG TCC ATG GAA GCA GTG AAC AAG AAG GCA CGT GTG GTT AAA       528
Leu Ser Thr Ser Met Glu Ala Val Asn Lys Lys Ala Arg Val Val Lys
                150                 155                 160

GAC GAA GCT AGA TTC CTT CTT ATC GCT ATT CAG ATG ACG GCT GAG GCA       576
Asp Glu Ala Arg Phe Leu Leu Ile Ala Ile Gln Met Thr Ala Glu Ala
            165                 170                 175
```

```
GCG CGA TTT AGG TAC ATA CAA AAC TTG GTA ATC AAG AAC TTT CCC AAC      624
Ala Arg Phe Arg Tyr Ile Gln Asn Leu Val Ile Lys Asn Phe Pro Asn
        180                 185                 190

AAG TTC AAC TCG GAA AAC AAA GTG ATT CAG TTT GAG GTT AAC TGG AAA      672
Lys Phe Asn Ser Glu Asn Lys Val Ile Gln Phe Glu Val Asn Trp Lys
    195                 200                 205

AAA ATT TCT ACG GCA ATA TAC GGG GAT GCC AAA AAC GGC GTG TTT AAT      720
Lys Ile Ser Thr Ala Ile Tyr Gly Asp Ala Lys Asn Gly Val Phe Asn
210                 215                 220                 225

AAA GAT TAT GAT TTC GGG TTT GGA AAA GTG AGG CAG GTG AAG GAC TTG      768
Lys Asp Tyr Asp Phe Gly Phe Gly Lys Val Arg Gln Val Lys Asp Leu
                230                 235                 240

CAA ATG GGA CTC CTT ATG TAT TTG GGC AAA CCA AAG                      804
Gln Met Gly Leu Leu Met Tyr Leu Gly Lys Pro Lys
            245                 250
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 10..15
(D) OTHER INFORMATION: /standard_name= "Nco I restriction enzyme recognition site"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 15..22
(D) OTHER INFORMATION: /product= "N-terminus of Saporin protein"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAACAACTGC CATGGTCACA TC 22

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 11..16
(D) OTHER INFORMATION: /standard_name= "Nco I restriction enzyme recognition site."

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1..10
(D) OTHER INFORMATION: /product= "Carboxy terminus of mature FGF protein"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCTAAGAGCG CCATGGAGA 19

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /product= "Carboxy terminus of wild type FGF"

(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 13..18
(D) OTHER INFORMATION: /standard_name= "Nco I restriction enzyme recognition site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCT AAG AGC TGACCATGGA GA 21
Ala Lys Ser
1

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 102 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..96
(D) OTHER INFORMATION: /product= "pFGFNcoI"
/note= "Equals the plasmid pFC80 wih native FGF stop codon removed."

(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 29..34
(D) OTHER INFORMATION: /standard_name= "Nco I restriction enzyme recognition site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
CTT TTT CTT CCA ATG TCT GCT AAG AGC GCC ATG GAG ATC CGG CTG AAT        48
Leu Phe Leu Pro Met Ser Ala Lys Ser Ala Met Glu Ile Arg Leu Asn
  1               5                  10                  15

GGT GCA GTT CTG TAC CGG TTT TCC TGT GCC GTC TTT CAG GAC TCC TGAAATCTT
102
Gly Ala Val Leu Tyr Arg Phe Ser Cys Ala Val Phe Gln Asp Ser
             20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1230 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1230

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1..465
(D) OTHER INFORMATION: /product= "bFGF"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 472..1230
(D) OTHER INFORMATION: /product= "Saporin"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
ATG GCA GCA GGA TCA ATA ACA ACA TTA CCC GCC TTG CCC GAG GAT GGC        48
Met Ala Ala Gly Ser Ile Thr Thr Leu Pro Ala Leu Pro Glu Asp Gly
  1               5                  10                  15

GGC AGC GGC GCC TTC CCG CCC GGC CAC TTC AAG GAC CCC AAG CGG CTG        96
Gly Ser Gly Ala Phe Pro Pro Gly His Phe Lys Asp Pro Lys Arg Leu
             20                  25                  30

TAC TGC AAA AAC GGG GGC TTC TTC CTG CGC ATC CAC CCC GAC GGC CGA       144
Tyr Cys Lys Asn Gly Gly Phe Phe Leu Arg Ile His Pro Asp Gly Arg
         35                  40                  45

GTT GAC GGG GTC CGG GAG AAG AGC GAC CCT CAC ATC AAG CTT CAA CTT       192
Val Asp Gly Val Arg Glu Lys Ser Asp Pro His Ile Lys Leu Gln Leu
     50                  55                  60

CAA GCA GAA GAG AGA GGA GTT GTG TCT ATC AAA GGA GTG TGT GCT AAC       240
Gln Ala Glu Glu Arg Gly Val Val Ser Ile Lys Gly Val Cys Ala Asn
 65                  70                  75                  80

CGT TAC CTG GCT ATG AAG GAA GAT GGA AGA TTA CTG GCT TCT AAA TGT       288
Arg Tyr Leu Ala Met Lys Glu Asp Gly Arg Leu Leu Ala Ser Lys Cys
                 85                  90                  95

GTT ACG GAT GAG TGT TTC TTT TTT GAA CGA TTG GAA TCT AAT AAC TAC       336
Val Thr Asp Glu Cys Phe Phe Phe Glu Arg Leu Glu Ser Asn Asn Tyr
            100                 105                 110
```

```
AAT ACT TAC CGG TCA AGG AAA TAC ACC AGT TGG TAT GTG GCA TTG AAA        384
Asn Thr Tyr Arg Ser Arg Lys Tyr Thr Ser Trp Tyr Val Ala Leu Lys
        115                 120                 125

CGA ACT GGG CAG TAT AAA CTT GGA TCC AAA ACA GGA CCT GGG CAG AAA        432
Arg Thr Gly Gln Tyr Lys Leu Gly Ser Lys Thr Gly Pro Gly Gln Lys
    130                 135                 140

GCT ATA CTT TTT CTT CCA ATG TCT GCT AAG AGC GCC ATG GTC ACA TCA        480
Ala Ile Leu Phe Leu Pro Met Ser Ala Lys Ser Ala Met Val Thr Ser
145                 150                 155                 160

ATC ACA TTA GAT CTA GTA AAT CCG ACC GCG GGT CAA TAC TCA TCT TTT        528
Ile Thr Leu Asp Leu Val Asn Pro Thr Ala Gly Gln Tyr Ser Ser Phe
                165                 170                 175

GTG GAT AAA ATC CGA AAC AAC GTA AAG GAT CCA AAC CTG AAA TAC GGT        576
Val Asp Lys Ile Arg Asn Asn Val Lys Asp Pro Asn Leu Lys Tyr Gly
            180                 185                 190

GGT ACC GAC ATA GCC GTG ATA GGC CCA CCT TCT AAA GAA AAA TTC CTT        624
Gly Thr Asp Ile Ala Val Ile Gly Pro Pro Ser Lys Glu Lys Phe Leu
        195                 200                 205

AGA ATT AAT TTC CAA AGT TCC CGA GGA ACG GTC TCA CTT GGC CTA AAA        672
Arg Ile Asn Phe Gln Ser Ser Arg Gly Thr Val Ser Leu Gly Leu Lys
    210                 215                 220

CGC GAT AAC TTG TAT GTG GTC GCG TAT CTT GCA ATG GAT AAC ACG AAT        720
Arg Asp Asn Leu Tyr Val Val Ala Tyr Leu Ala Met Asp Asn Thr Asn
225                 230                 235                 240

GTT AAT CGG GCA TAT TAC TTC AAA TCA GAA ATT ACT TCC GCC GAG TTA        768
Val Asn Arg Ala Tyr Tyr Phe Lys Ser Glu Ile Thr Ser Ala Glu Leu
                245                 250                 255

ACC GCC CTT TTC CCA GAG GCC ACA ACT GCA AAT CAG AAA GCT TTA GAA        816
Thr Ala Leu Phe Pro Glu Ala Thr Thr Ala Asn Gln Lys Ala Leu Glu
            260                 265                 270

TAC ACA GAA GAT TAT CAG TCG ATC GAA AAG AAT GCC CAG ATA ACA CAG        864
Tyr Thr Glu Asp Tyr Gln Ser Ile Glu Lys Asn Ala Gln Ile Thr Gln
        275                 280                 285

GGA GAT AAA AGT AGA AAA GAA CTC GGG TTG GGG ATC GAC TTA CTT TTG        912
Gly Asp Lys Ser Arg Lys Glu Leu Gly Leu Gly Ile Asp Leu Leu Leu
    290                 295                 300

ACG TTC ATG GAA GCA GTG AAC AAG AAG GCA CGT GTG GTT AAA AAC GAA        960
Thr Phe Met Glu Ala Val Asn Lys Lys Ala Arg Val Val Lys Asn Glu
305                 310                 315                 320

GCT AGG TTT CTG CTT ATC GCT ATT CAA ATG ACA GCT GAG GTA GCA CGA       1008
Ala Arg Phe Leu Leu Ile Ala Ile Gln Met Thr Ala Glu Val Ala Arg
                325                 330                 335

TTT AGG TAC ATT CAA AAC TTG GTA ACT AAG AAC TTC CCC AAC AAG TTC       1056
Phe Arg Tyr Ile Gln Asn Leu Val Thr Lys Asn Phe Pro Asn Lys Phe
            340                 345                 350
```

```
GAC TCG GAT AAC AAG GTG ATT CAA TTT GAA GTC AGC TGG CGT AAG ATT      1104
Asp Ser Asp Asn Lys Val Ile Gln Phe Glu Val Ser Trp Arg Lys Ile
        355                 360                 365

TCT ACG GCA ATA TAC GGG GAT GCC AAA AAC GGC GTG TTT AAT AAA GAT      1152
Ser Thr Ala Ile Tyr Gly Asp Ala Lys Asn Gly Val Phe Asn Lys Asp
    370                 375                 380

TAT GAT TTC GGG TTT GGA AAA GTG AGG CAG GTG AAG GAC TTG CAA ATG      1200
Tyr Asp Phe Gly Phe Gly Lys Val Arg Gln Val Lys Asp Leu Gln Met
385                 390                 395                 400

GGA CTC CTT ATG TAT TTG GGC AAA CCA AAG                              1230
Gly Leu Leu Met Tyr Leu Gly Lys Pro Lys
                405                 410
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1230 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1230

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1..465
(D) OTHER INFORMATION: /product= "bFGF"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 472..1230
(D) OTHER INFORMATION: /product= "Saporin"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
ATG GCT GCT GGT TCT ATC ACT ACT CTG CCG GCT CTG CCG GAA GAC GGT        48
Met Ala Ala Gly Ser Ile Thr Thr Leu Pro Ala Leu Pro Glu Asp Gly
  1               5                  10                  15

GGT TCT GGT GCT TTC CCG CCC GGC CAC TTC AAG GAC CCC AAG CGG CTG        96
Gly Ser Gly Ala Phe Pro Pro Gly His Phe Lys Asp Pro Lys Arg Leu
            20                  25                  30

TAC TGC AAA AAC GGG GGC TTC TTC CTG CGC ATC CAC CCC GAC GGC CGA       144
Tyr Cys Lys Asn Gly Gly Phe Phe Leu Arg Ile His Pro Asp Gly Arg
        35                  40                  45

GTT GAC GGG GTC CGG GAG AAG AGC GAC CCT CAC ATC AAG CTT CAA CTT       192
Val Asp Gly Val Arg Glu Lys Ser Asp Pro His Ile Lys Leu Gln Leu
    50                  55                  60

CAA GCA GAA GAG AGA GGA GTT GTG TCT ATC AAA GGA GTG TGT GCT AAC       240
Gln Ala Glu Glu Arg Gly Val Val Ser Ile Lys Gly Val Cys Ala Asn
65                  70                  75                  80
```

```
CGT TAC CTG GCT ATG AAG GAA GAT GGA AGA TTA CTG GCT TCT AAA TGT        288
Arg Tyr Leu Ala Met Lys Glu Asp Gly Arg Leu Leu Ala Ser Lys Cys
                 85                  90                  95

GTT ACG GAT GAG TGT TTC TTT TTT GAA CGA TTG GAA TCT AAT AAC TAC        336
Val Thr Asp Glu Cys Phe Phe Phe Glu Arg Leu Glu Ser Asn Asn Tyr
            100                 105                 110

AAT ACT TAC CGG TCA AGG AAA TAC ACC AGT TGG TAT GTG GCA TTG AAA        384
Asn Thr Tyr Arg Ser Arg Lys Tyr Thr Ser Trp Tyr Val Ala Leu Lys
        115                 120                 125

CGA ACT GGG CAG TAT AAA CTT GGA TCC AAA ACA GGA CCT GGG CAG AAA        432
Arg Thr Gly Gln Tyr Lys Leu Gly Ser Lys Thr Gly Pro Gly Gln Lys
    130                 135                 140

GCT ATA CTT TTT CTT CCA ATG TCT GCT AAG AGC GCC ATG GTC ACA TCA        480
Ala Ile Leu Phe Leu Pro Met Ser Ala Lys Ser Ala Met Val Thr Ser
145                 150                 155                 160

ATC ACA TTA GAT CTA GTA AAT CCG ACC GCG GGT CAA TAC TCA TCT TTT        528
Ile Thr Leu Asp Leu Val Asn Pro Thr Ala Gly Gln Tyr Ser Ser Phe
                165                 170                 175

GTG GAT AAA ATC CGA AAC AAC GTA AAG GAT CCA AAC CTG AAA TAC GGT        576
Val Asp Lys Ile Arg Asn Asn Val Lys Asp Pro Asn Leu Lys Tyr Gly
            180                 185                 190

GGT ACC GAC ATA GCC GTG ATA GGC CCA CCT TCT AAA GAA AAA TTC CTT        624
Gly Thr Asp Ile Ala Val Ile Gly Pro Pro Ser Lys Glu Lys Phe Leu
        195                 200                 205

AGA ATT AAT TTC CAA AGT TCC CGA GGA ACG GTC TCA CTT GGC CTA AAA        672
Arg Ile Asn Phe Gln Ser Ser Arg Gly Thr Val Ser Leu Gly Leu Lys
    210                 215                 220

CGC GAT AAC TTG TAT GTG GTC GCG TAT CTT GCA ATG GAT AAC ACG AAT        720
Arg Asp Asn Leu Tyr Val Val Ala Tyr Leu Ala Met Asp Asn Thr Asn
225                 230                 235                 240

GTT AAT CGG GCA TAT TAC TTC AAA TCA GAA ATT ACT TCC GCC GAG TTA        768
Val Asn Arg Ala Tyr Tyr Phe Lys Ser Glu Ile Thr Ser Ala Glu Leu
                245                 250                 255

ACC GCC CTT TTC CCA GAG GCC ACA ACT GCA AAT CAG AAA GCT TTA GAA        816
Thr Ala Leu Phe Pro Glu Ala Thr Thr Ala Asn Gln Lys Ala Leu Glu
            260                 265                 270

TAC ACA GAA GAT TAT CAG TCG ATC GAA AAG AAT GCC CAG ATA ACA CAG        864
Tyr Thr Glu Asp Tyr Gln Ser Ile Glu Lys Asn Ala Gln Ile Thr Gln
        275                 280                 285

GGA GAT AAA AGT AGA AAA GAA CTC GGG TTG GGG ATC GAC TTA CTT TTG        912
Gly Asp Lys Ser Arg Lys Glu Leu Gly Leu Gly Ile Asp Leu Leu Leu
    290                 295                 300

ACG TTC ATG GAA GCA GTG AAC AAG AAG GCA CGT GTG GTT AAA AAC GAA        960
Thr Phe Met Glu Ala Val Asn Lys Lys Ala Arg Val Val Lys Asn Glu
305                 310                 315                 320
```

```
GCT AGG TTT CTG CTT ATC GCT ATT CAA ATG ACA GCT GAG GTA GCA CGA      1008
Ala Arg Phe Leu Leu Ile Ala Ile Gln Met Thr Ala Glu Val Ala Arg
                325                 330                 335

TTT AGG TAC ATT CAA AAC TTG GTA ACT AAG AAC TTC CCC AAC AAG TTC      1056
Phe Arg Tyr Ile Gln Asn Leu Val Thr Lys Asn Phe Pro Asn Lys Phe
            340                 345                 350

GAC TCG GAT AAC AAG GTG ATT CAA TTT GAA GTC AGC TGG CGT AAG ATT      1104
Asp Ser Asp Asn Lys Val Ile Gln Phe Glu Val Ser Trp Arg Lys Ile
        355                 360                 365

TCT ACG GCA ATA TAC GGG GAT GCC AAA AAC GGC GTG TTT AAT AAA GAT      1152
Ser Thr Ala Ile Tyr Gly Asp Ala Lys Asn Gly Val Phe Asn Lys Asp
    370                 375                 380

TAT GAT TTC GGG TTT GGA AAA GTG AGG CAG GTG AAG GAC TTG CAA ATG      1200
Tyr Asp Phe Gly Phe Gly Lys Val Arg Gln Val Lys Asp Leu Gln Met
385                 390                 395                 400

GGA CTC CTT ATG TAT TTG GGC AAA CCA AAG                              1230
Gly Leu Leu Met Tyr Leu Gly Lys Pro Lys
                405                 410
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
AATTCCCCTG TTGACAATTA ATCATCGAAC TAGTTAACTA GTACGCAGCT TGGCTGCAG      59
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
GTCGACCAAG CTTGGGCATA CATTCAATCA ATTGTTATCT AAGGAAATAC TTACATATG      59
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGGAGTGTCT GCTAACC 17

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTCTAAATCG GTTACCGATG ACTG 24

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAATACTTAC ATATGGCAGC AGGATC 26

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CAGGTAACGG TTAGCAGACA CTCCTTTGAT 30

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATCAAAGGAG TGTCTGCTAA CCGTTACCTG 30

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTGATTGATG TGACCATGGC GCTCTTAGCA 30

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TGGCTTCTAA ATCTGTTACG GATGAG 26

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCATCCGTA ACAGATTTAG AAGCCA 26

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 155 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Met Ala Glu Gly Glu Ile Thr Thr Phe Thr Ala Leu Thr Glu Lys Phe
1 5 10 15

Asn Leu Pro Pro Gly Asn Tyr Lys Lys Pro Lys Leu Leu Tyr Cys Ser
20 25 30

Asn Gly Gly His Phe Leu Arg Ile Leu Pro Asp Gly Thr Val Asp Gly
35 40 45

Thr Arg Asp Arg Ser Asp Gln His Ile Gln Leu Gln Leu Ser Ala Glu
50 55 60

Ser Val Gly Glu Val Tyr Ile Lys Ser Thr Glu Thr Gly Gln Tyr Leu
65 70 75 80

Ala Met Asp Thr Asp Gly Leu Leu Tyr Gly Ser Gln Thr Pro Asn Glu
85 90 95

Glu Cys Leu Phe Leu Glu Arg Leu Glu Glu Asn His Tyr Asn Thr Tyr
100 105 110

Ile Ser Lys Lys His Ala Glu Lys Asn Trp Phe Val Gly Leu Lys Lys
115 120 125

Asn Gly Ser Cys Lys Arg Gly Pro Arg Thr His Tyr Gly Gln Lys Ala
130 135 140

Ile Leu Phe Leu Pro Leu Pro Val Ser Ser Asp
145 150 155

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 155 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Ala Ala Gly Ser Ile Thr Thr Leu Pro Ala Leu Pro Glu Asp Gly
1 5 10 15

Gly Ser Gly Ala Phe Pro Pro Gly His Phe Lys Asp Pro Lys Arg Leu
20 25 30

Tyr Cys Lys Asn Gly Gly Phe Phe Leu Arg Ile His Pro Asp Gly Arg
35 40 45

Val Asp Gly Val Arg Glu Lys Ser Asp Pro His Ile Lys Leu Gln Leu
50 55 60

Gln Ala Glu Glu Arg Gly Val Val Ser Ile Lys Gly Val Cys Ala Asn
65 70 75 80

Arg Tyr Leu Ala Met Lys Glu Asp Gly Arg Leu Leu Ala Ser Lys Cys
85 90 95

Val Thr Asp Glu Cys Phe Phe Phe Glu Arg Leu Glu Ser Asn Asn Tyr
100 105 110

Asn Thr Tyr Arg Ser Arg Lys Tyr Thr Ser Trp Tyr Val Ala Leu Lys
115 120 125

```
Arg Thr Gly Gln Tyr Lys Leu Gly Ser Lys Thr Gly Pro Gly Gln Lys
    130                 135                 140

Ala Ile Leu Phe Leu Pro Met Ser Ala Lys Ser
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 239 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
Met Gly Leu Ile Trp Leu Leu Leu Leu Ser Leu Leu Glu Pro Gly Trp
1               5                   10                  15

Pro Ala Ala Gly Pro Gly Ala Arg Leu Arg Arg Asp Ala Gly Gly Arg
            20                  25                  30

Gly Gly Val Tyr Glu His Leu Gly Gly Ala Pro Arg Arg Arg Lys Leu
        35                  40                  45

Tyr Cys Ala Thr Lys Tyr His Leu Gln Leu His Pro Ser Gly Arg Val
    50                  55                  60

Asn Gly Ser Leu Glu Asn Ser Ala Tyr Ser Ile Leu Glu Ile Thr Ala
65                  70                  75                  80

Val Glu Val Gly Ile Val Ala Ile Arg Gly Leu Phe Ser Gly Arg Tyr
                85                  90                  95

Leu Ala Met Asn Lys Arg Gly Arg Leu Tyr Ala Ser Glu His Tyr Ser
            100                 105                 110

Ala Glu Cys Glu Phe Val Glu Arg Ile His Glu Leu Gly Tyr Asn Thr
        115                 120                 125

Tyr Ala Ser Arg Leu Tyr Arg Thr Val Ser Ser Thr Pro Gly Ala Arg
    130                 135                 140

Arg Gln Pro Ser Ala Glu Arg Leu Trp Tyr Val Ser Val Asn Gly Lys
145                 150                 155                 160

Gly Arg Pro Arg Arg Gly Phe Lys Thr Arg Arg Thr Gln Lys Ser Ser
                165                 170                 175

Leu Phe Leu Pro Arg Val Leu Asp His Arg Asp His Glu Met Val Arg
            180                 185                 190

Gln Leu Gln Ser Gly Leu Pro Arg Pro Pro Gly Lys Gly Val Gln Pro
        195                 200                 205

Arg Arg Arg Arg Gln Lys Gln Ser Pro Asp Asn Leu Glu Pro Ser His
    210                 215                 220

Val Gln Ala Ser Arg Leu Gly Ser Gln Leu Glu Ala Ser Ala His
225                 230                 235
```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 206 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
Met Ser Gly Pro Gly Thr Ala Ala Val Ala Leu Leu Pro Ala Val Leu
1               5                  10                  15

Leu Ala Leu Leu Ala Pro Trp Ala Gly Arg Gly Gly Ala Ala Ala Pro
            20                  25                  30

Thr Ala Pro Asn Gly Thr Leu Glu Ala Glu Leu Glu Arg Arg Trp Glu
        35                  40                  45

Ser Leu Val Ala Leu Ser Leu Ala Arg Leu Pro Val Ala Ala Gln Pro
    50                  55                  60

Lys Glu Ala Ala Val Gln Ser Gly Ala Gly Asp Tyr Leu Leu Gly Ile
65                  70                  75                  80

Lys Arg Leu Arg Arg Leu Tyr Cys Asn Val Gly Ile Gly Phe His Leu
                85                  90                  95

Gln Ala Leu Pro Asp Gly Arg Ile Gly Gly Ala His Ala Asp Thr Arg
            100                 105                 110

Asp Ser Leu Leu Glu Leu Ser Pro Val Glu Arg Gly Val Val Ser Ile
        115                 120                 125

Phe Gly Val Ala Ser Arg Phe Phe Val Ala Met Ser Ser Lys Gly Lys
    130                 135                 140

Leu Tyr Gly Ser Pro Phe Phe Thr Asp Glu Cys Thr Phe Lys Glu Ile
145                 150                 155                 160

Leu Leu Pro Asn Asn Tyr Asn Ala Tyr Glu Ser Tyr Lys Tyr Pro Gly
                165                 170                 175

Met Phe Ile Ala Leu Ser Lys Asn Gly Lys Thr Lys Lys Gly Asn Arg
            180                 185                 190

Val Ser Pro Thr Met Lys Val Thr His Phe Leu Pro Arg Leu
        195                 200                 205
```

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 268 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
Met Ser Leu Ser Phe Leu Leu Leu Leu Phe Phe Ser His Leu Ile Leu
1               5                  10                  15

Ser Ala Trp Ala His Gly Glu Lys Arg Leu Ala Pro Lys Gly Gln Pro
            20                  25                  30

Gly Pro Ala Ala Thr Asp Arg Asn Pro Ile Gly Ser Ser Ser Arg Gln
        35                  40                  45

Ser Ser Ser Ser Ala Met Ser Ser Ser Ser Ala Ser Ser Ser Pro Ala
    50                  55                  60

Ala Ser Leu Gly Ser Gln Gly Ser Gly Leu Glu Gln Ser Ser Phe Gln
65                  70                  75                  80

Trp Ser Pro Ser Gly Arg Arg Thr Gly Ser Leu Tyr Cys Arg Val Gly
                85                  90                  95

Ile Gly Phe His Leu Gln Ile Tyr Pro Asp Gly Lys Val Asn Gly Ser
            100                 105                 110

His Glu Ala Asn Met Leu Ser Val Leu Glu Ile Phe Ala Val Ser Gln
        115                 120                 125

Gly Ile Val Gly Ile Arg Gly Val Phe Ser Asn Lys Phe Leu Ala Met
    130                 135                 140

Ser Lys Lys Gly Lys Leu His Ala Ser Ala Lys Phe Thr Asp Asp Cys
145                 150                 155                 160

Lys Phe Arg Glu Arg Phe Gln Glu Asn Ser Tyr Asn Thr Tyr Ala Ser
                165                 170                 175

Ala Ile His Arg Thr Glu Lys Thr Gly Arg Glu Trp Tyr Val Ala Leu
            180                 185                 190

Asn Lys Arg Gly Lys Ala Lys Arg Gly Cys Ser Pro Arg Val Lys Pro
        195                 200                 205

Gln His Ile Ser Thr His Phe Leu Pro Arg Phe Lys Gln Ser Glu Gln
    210                 215                 220

Pro Glu Leu Ser Phe Thr Val Thr Val Pro Glu Lys Lys Asn Pro Pro
225                 230                 235                 240

Ser Pro Ile Lys Ser Lys Ile Pro Leu Ser Ala Pro Arg Lys Asn Thr
                245                 250                 255

Asn Ser Val Lys Tyr Arg Leu Lys Phe Arg Phe Gly
            260                 265
```

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 198 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
Met Ser Arg Gly Ala Gly Arg Leu Gln Gly Thr Leu Trp Ala Leu Val
1               5                  10                  15

Phe Leu Gly Ile Leu Val Gly Met Val Val Pro Ser Pro Ala Gly Thr
            20                  25                  30

Arg Ala Asn Asn Thr Leu Leu Asp Ser Arg Gly Trp Gly Thr Leu Leu
        35                  40                  45

Ser Arg Ser Arg Ala Gly Leu Ala Gly Glu Ile Ala Gly Val Asn Trp
    50                  55                  60

Glu Ser Gly Tyr Leu Val Gly Ile Lys Arg Gln Arg Arg Leu Tyr Cys
65                  70                  75                  80

Asn Val Gly Ile Gly Phe His Leu Gln Val Leu Pro Asp Gly Arg Ile
                85                  90                  95

Ser Gly Thr His Glu Glu Asn Pro Tyr Ser Leu Leu Glu Ile Ser Thr
            100                 105                 110

Val Glu Arg Gly Val Val Ser Leu Phe Gly Val Arg Ser Ala Leu Phe
        115                 120                 125

Val Ala Met Asn Ser Lys Gly Arg Leu Tyr Ala Thr Pro Ser Phe Gln
    130                 135                 140

Glu Glu Cys Lys Phe Arg Glu Thr Leu Leu Pro Asn Asn Tyr Asn Ala
145                 150                 155                 160

Tyr Glu Ser Asp Leu Tyr Gln Gly Thr Tyr Ile Ala Leu Ser Lys Tyr
                165                 170                 175

Gly Arg Val Lys Arg Gly Ser Lys Val Ser Pro Ile Met Thr Val Thr
            180                 185                 190

His Phe Leu Pro Arg Ile
        195
```

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 194 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```
Met His Lys Trp Ile Leu Thr Trp Ile Leu Pro Thr Leu Leu Tyr Arg
1               5                  10                  15

Ser Cys Phe His Ile Ile Cys Leu Val Gly Thr Ile Ser Leu Ala Cys
            20                  25                  30

Asn Asp Met Thr Pro Glu Gln Met Ala Thr Asn Val Asn Cys Ser Ser
        35                  40                  45

Pro Glu Arg His Thr Arg Ser Tyr Asp Tyr Met Glu Gly Gly Asp Ile
    50                  55                  60
```

```
Arg Val Arg Arg Leu Phe Cys Arg Thr Gln Trp Tyr Leu Arg Ile Asp
65                  70                  75                  80

Lys Arg Gly Lys Val Lys Gly Thr Gln Glu Met Lys Asn Asn Tyr Asn
                85                  90                  95

Ile Met Glu Ile Arg Thr Val Ala Val Gly Ile Val Ala Ile Lys Gly
            100                 105                 110

Val Glu Ser Glu Phe Tyr Leu Ala Met Asn Lys Glu Gly Lys Leu Tyr
        115                 120                 125

Ala Lys Lys Glu Cys Asn Glu Asp Cys Asn Phe Lys Glu Leu Ile Leu
    130                 135                 140

Glu Asn His Tyr Asn Thr Tyr Ala Ser Ala Lys Trp Thr His Asn Gly
145                 150                 155                 160

Gly Glu Met Phe Val Ala Leu Asn Gln Lys Gly Ile Pro Val Arg Gly
                165                 170                 175

Lys Lys Thr Lys Lys Glu Gln Lys Thr Ala His Phe Leu Pro Met Ala
            180                 185                 190

Ile Thr
```

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 215 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```
Met Gly Ser Pro Arg Ser Ala Leu Ser Cys Leu Leu Leu His Leu Leu
1               5                   10                  15

Val Leu Cys Leu Gln Ala Gln Val Thr Val Gln Ser Ser Pro Asn Phe
            20                  25                  30

Thr Gln His Val Arg Glu Gln Ser Leu Val Thr Asp Gln Leu Ser Arg
        35                  40                  45

Arg Leu Ile Arg Thr Tyr Gln Leu Tyr Ser Arg Thr Ser Gly Lys His
    50                  55                  60

Val Gln Val Leu Ala Asn Lys Arg Ile Asn Ala Met Ala Glu Asp Gly
65                  70                  75                  80

Asp Pro Phe Ala Lys Leu Ile Val Glu Thr Asp Thr Phe Gly Ser Arg
                85                  90                  95

Val Arg Val Arg Gly Ala Glu Thr Gly Leu Tyr Ile Cys Met Asn Lys
            100                 105                 110

Lys Gly Lys Leu Ile Ala Lys Ser Asn Gly Lys Gly Lys Asp Cys Val
        115                 120                 125
```

Phe Thr Glu Ile Val Leu Glu Asn Asn Tyr Thr Ala Leu Gln Asn Ala
130 135 140

Lys Tyr Glu Gly Trp Tyr Met Ala Phe Thr Arg Lys Gly Arg Pro Arg
145 150 155 160

Lys Gly Ser Lys Thr Arg Gln His Gln Arg Glu Val His Phe Met Lys
165 170 175

Arg Leu Pro Arg Gly His His Thr Thr Glu Gln Ser Leu Arg Phe Glu
180 185 190

Phe Leu Asn Tyr Pro Pro Phe Thr Arg Ser Leu Arg Gly Ser Gln Arg
195 200 205

Thr Trp Ala Pro Glu Pro Arg
210 215

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 208 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Met Ala Pro Leu Gly Glu Val Gly Asn Tyr Phe Gly Val Gln Asp Ala
1 5 10 15

Val Pro Phe Gly Asn Val Pro Val Leu Pro Val Asp Ser Pro Val Leu
20 25 30

Leu Ser Asp His Leu Gly Gln Ser Glu Ala Gly Gly Leu Pro Arg Gly
35 40 45
Pro Ala Val Thr Asp Leu Asp His Leu Lys Gly Ile Leu Arg Arg Arg
50 55 60

Gln Leu Tyr Cys Arg Thr Gly Phe His Leu Glu Ile Phe Pro Asn Gly
65 70 75 80

Thr Ile Gln Gly Thr Arg Lys Asp His Ser Arg Phe Gly Ile Leu Glu
85 90 95

Phe Ile Ser Ile Ala Val Gly Leu Val Ser Ile Arg Gly Val Asp Ser
100 105 110

Gly Leu Tyr Leu Gly Met Asn Glu Lys Gly Glu Leu Tyr Gly Ser Glu
115 120 125

Lys Leu Thr Gln Glu Cys Val Phe Arg Glu Gln Phe Glu Glu Asn Trp
130 135 140

Tyr Asn Thr Tyr Ser Ser Asn Leu Tyr Lys His Val Asp Thr Gly Arg
145 150 155 160

Arg Tyr Tyr Val Ala Leu Asn Lys Asp Gly Thr Pro Arg Glu Gly Thr
165 170 175

Arg Thr Lys Arg His Gln Lys Phe Thr His Phe Leu Pro Arg Pro Val
180 185 190

Asp Pro Asp Lys Val Pro Glu Leu Tyr Lys Asp Ile Leu Ser Gln Ser
195 200 205

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Val Ile Ile Tyr Glu Leu Asn Leu Gln Gly Thr Thr Lys Ala Gln Tyr
5 10 15

Ser Thr Ile Leu Lys Gln Leu Arg Asp Asp Ile Lys Asp Pro Asn Leu
20 25 30

Xaa Tyr Gly Xaa Xaa Asp Tyr Ser
35 40

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34

CATATGTGTG TCACATCAAT CACATTAGAT 30

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35

CAGGTTTGGA TCCTTTACGT T 21

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 82 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36

```
AAGGAGATATACC ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC          43
              Met Gly Ser Ser His His His His His His Ser Ser
               1               5                  10

GGC CTG GTG CCG CGC GGC AGC CAT ATG CTC GAG GAT CCG                    82
Gly Leu Val Pro Arg Gly Ser His Met Leu Glu Asp Pro
        15                  20                  25
```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37

```
GGATCCGCCT CGTTTGACTA CTT                                              23
```

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38

```
CATATGGTCA CATCATGTAC ATTAGATCTA GTAAAT                                36
```

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39

```
CATATGGTCA CATCAATCAC ATTAGATCTA GTATGTCCGA CCGCGGGTCA                 50
```

CLAIMS:

1. A monogenous preparation of cytotoxic conjugates, comprising cytotoxic conjugates that contain a cytotoxic agent and a polypeptide reactive with a fibroblast growth factor (FGF) receptor, wherein:

the cytotoxic conjugate binds to an FGF receptor and internalizes the cytotoxic agent in cells bearing the FGF receptor; and

substantially all of the cytotoxic conjugates in the monogenous preparation contain the same molar ratio of cytotoxic agent to polypeptide reactive with an FGF receptor.

2. The preparation of claim 1, wherein the conjugate is a chemical conjugate or a fusion protein.

3. The preparation of claim 1 or claim 2, wherein the conjugate has the formula:

$(FGF)_n$-(cytotoxic agent)$_m$, wherein:

FGF is a polypeptide reactive with a fibroblast growth factor (FGF) receptor;

the conjugate binds to an FGF receptor and internalizes the cytotoxic agent in cells bearing an FGF receptor;

n and m, which are the same or different, are 1 to 4; and

if m or n, or m and n are greater than 1, then the conjugate contains up to m different cytotoxic agents and up to n FGF polypeptides.

4. The preparation of claim 3 in which the conjugate is represented by the formula FGF-Ala-Met-SAP-Ala-Met-SAP, in which the FGF has been modified by replacement or deletion of one or more cysteine residues.

5. The preparation of claim 4, wherein the FGF is basic FGF and the cysteine residues at position 78 or 96 or both is (are) replaced with serine.

6. The monogenous preparation of any of claims 1-4, wherein the polypeptide reactive with an FGF receptor is basic FGF that has been modified by replacement of the cysteine residue at position 78 or 96 with a serine residue or by replacement of the cysteine residues at positions 78 and

96 with serine residues; and the position numbers are determined by reference to SEQ ID NO. 24.

7. The preparation of any of claims 1-4, wherein:

the polypeptide reactive with an FGF receptor has been modified by replacement of cysteine residues with serine such that the resulting polypeptide reactive with an FGF receptor has at least two cysteines and retains the ability to bind to an FGF receptor and internalize the linked cytotoxic agent;

the polypeptide reactive with an FGF receptor is selected from the group consisting of FGF-1, FGF-5, FGF-7 and FGF-8;

the FGF-1 has been modified by replacement of the cysteine residues at position 31 or 132 with serine;

the FGF-5 has been modified by replacement of the cysteine residues at position 19, 93, or 202 or at least two of positions 19, 93, or 202 with serine;

FGF-7 has been modified by replacement of the cysteine residues at position 18, 23, 32, 46, 71 or 133, or at least two of positions 18, 23, 32, 46, 71 or 133, or at least three of positions 18, 23, 32, 46, 71 or 133, or at least four of positions 18, 23, 32, 46, 71 or 133, or at least five of positions 18, 23, 32, 46, 71 or 133 with serine;

FGF-8 has been modified by replacement of the cysteine residues at position 10, 19, 109 or 127, or at least two of positions 10, 19, 109 or 127, or at least three of positions 10, 19, 109 and 127 with serine; and

the position numbers are determined by reference to SEQ ID NO. 24 for FGF-1; SEQ ID NO. 28 for FGF-5; SEQ ID NO. 30 for FGF-7 and SEQ ID NO. 31 for FGF-8.

8. The preparation of any of claims 1-7, wherein the cytotoxic agent is a ribosome-inactivating protein.

9. The preparation of any of claims 1-8, wherein the cytotoxic agent is substantially pure mono-derivatized saporin.

10. The preparation of any of claims 1-8, wherein:

the cytotoxic agent is saporin that has been modified by the addition of a cysteine residue or replacement of a residue with a cysteine at or within about twenty amino acid residues of the N-terminus; and

the resulting modified saporin is, upon internalization by a eukaryotic cell, cytotoxic to the eukaryotic cell.

11. The preparation of claim 10, wherein the saporin is FPS1, FPS2 or FPS3.

12. The preparation of any of claims 1-8, wherein the cytotoxic agent(s) is (are) selected from methotrexate, anthracycline and Pseudomonas exotoxin.

13. A composition, comprising the monogenous preparation of cytotoxic conjugates of any of claims 1-12.

14. A pharmaceutical composition, comprising the monogenous preparation of any of claims 1-12 and a physiologically acceptable excipient.

15. A method of preparation of cytotoxic conjugates of claim 1, comprising reacting a mutein of a polypeptide reactive with a fibroblast growth factor (FGF) receptor with a cytotoxic agent to produce a monogenous preparation of cytotoxic conjugates, wherein the mutein polypeptide has been modified by replacement of one or more cysteine residues with another amino acid so that the resulting mutein has two or three cysteines and retains the ability to bind to an FGF receptor and internalize the linked cytotoxic agent; and

the cytotoxic agent either:

(i) contains only one cysteine;

(ii) is a single species of cytotoxic agent that has been derivatized to introduce a moiety that reacts with a cysteine residue on the polypeptide; or

(iii) has been modified by addition of a cysteine residue and the resulting modified agent contains only one cysteine.

16. The method of claim 15, wherein the polypeptide reactive with an FGF receptor is basic FGF; the cysteine residue that is replaced is Cys 78,

Cys 96 or Cys 78 and Cys 96; and the position numbers are determined by reference to SEQ ID NO. 24.

17. The method of claim 15, wherein the polypeptide reactive with an FGF receptor is selected from the group consisting of FGF-1, FGF-5, FGF-7 and FGF-8;

the FGF-1 has been modified by replacement of the cysteine residues at position 31 or 132 with serine;

the FGF-5 has been modified by replacement of the cysteine residues at position 19, 93, or 202 or at least two of positions 19, 93, or 202 with serine;

FGF-7 has been modified by replacement of the cysteine residues at position 18, 23, 32, 46, 71 or 133, or at least two of positions 18, 23, 32, 46, 71 or 133, or at least three of positions 18, 23, 32, 46, 71 or 133, or at least four of positions 18, 23, 32, 46, 71 or 133, or at least five of positions 18, 23, 32, 46, 71 or 133 with serine;

FGF-8 has been modified by replacement of the cysteine residues at position 10, 19, 109 or 127, or at least two of positions 10, 19, 109 or 127, or at least three of positions 10, 19, 109 and 127 with serine; and

the position numbers are determined by reference to SEQ ID NO. 24 for FGF-1; SEQ ID NO. 28 for FGF-5; SEQ ID NO. 30 for FGF-7 and SEQ ID NO. 31 for FGF-8.

18. The method of claim 15, wherein the cytotoxic agent is substantially pure mono-derivatized saporin.

19. The method of claim 15, wherein the cytotoxic agent is saporin that, prior to the reaction, is modified by addition of a cysteine residue at or within about twenty amino acid residues of the N-terminus, wherein the modified saporin is, upon internalization by a eukaryotic cell, cytotoxic to the eukaryotic cell.

20. The preparation of any of claims 1-8, wherein the cytotoxic agent is modified saporin that has been modified by addition of a cysteine residue at or within about twenty amino acid residues of the N-terminus; and

the modified saporin is, upon internalization by a eukaryotic cell, cytotoxic to the eukaryotic cell.

21. A cytotoxic conjugate, comprising a modified saporin and a polypeptide reactive with an FGF (fibroblast growth factor) receptor, wherein:

the polypeptide reactive with the FGF receptor binds to an FGF receptor and internalizes the cytotoxic agent in cells bearing the FGF receptor;

the saporin is modified to contain a cysteine residue at or substantially near the N-terminus; and

the modified saporin is, upon internalization by a eukaryotic cell, cytotoxic to the eukaryotic cell.

22. The conjugate of claim 21, wherein the polypeptide reactive with an FGF receptor is selected from the group consisting of FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8 and FGF-9.

23. The conjugate of claim 21 that is CCFS2, CCFS3 or CCFS4.

24. The preparation of claim 1, wherein each conjugate has the sequence set forth in SEQ ID NO. 12, except that the cysteine residue at position 78 or 96 has been replaced with a serine residue or the cysteine residues at positions 78 and 96 are replaced with serine residues.

25. The preparation of claim 24, wherein the conjugate is FPFS2, FPFS3, or FPFS4.

26. An isolated DNA fragment, comprising a sequence of nucleotides encoding a cytotoxic conjugate containing a modified polypeptide reactive with an FGF (FGF) receptor linked to a cytotoxic agent, wherein:

the polypeptide reactive with an FGF receptor has been modified by replacement of cysteine residues with serine such that the resulting polypeptide reactive with an FGF receptor has at least one cysteine and retains the ability to bind to an FGF receptor and internalize the linked cytotoxic agent;

the cytotoxic agent is linked via a linker peptide of n amino acids; and n is 0 to about 30.

27. The DNA fragment of claim 26, further comprising a promoter region and a transcription terminator region, wherein:

the promoter region includes an inducible promoter;

the promoter region and the transcription terminator are independently selected from the same or different genes and are operatively linked to the DNA encoding the saporin-containing protein.

28. The DNA fragment of claim 26, wherein the cytotoxic agent is saporin and the amino acid sequence of the saporin is set forth in SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6 or SEQ ID NO. 7.

29. The DNA fragment of any of claims 26-28, wherein the amino acid sequence of the FGF protein is set forth in SEQ ID NO. 12 or SEQ ID NO. 13, except that the cysteine residues at positions 78 and 96 are replaced with serines.

30. The DNA fragment of any of claims 26-29, further comprising DNA encoding a secretion signal sequence operatively linked to the DNA encoding the saporin-containing protein.

31. The DNA fragment of claim 30, wherein the secretion signal is ompA or ompT.

32. The DNA fragment of claim 27, wherein the promoter is the T7 promoter or the lacUV5 promoter.

33. The DNA fragment of any of claims 26-32, wherein the polypeptide reactive with an FGF receptor is selected from the group consisting of FGF-1, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8 and FGF-9, wherein:

FGF-1 has been modified by replacement of the cysteine residues at positions 31 or 132 or positions 31 and 132;

FGF-3 has been modified by replacement of the cysteine residue at p sition 50;

FGF-4 has been modified by replacement of the cysteine residue at 88;

FGF-5 has been modified by replacement of the cysteine residues at position 19, 93, or 202, or at least two of positions 19, 93, or 202, or at all of positions 19, 93, and 202;

FGF-6 has been modified by replacement of the cysteine at position 80;

FGF-7 has been modified by replacement of the cysteine residues at position 18, 23, 32, 46, 71 or 133, or at least two of positions 18, 23, 32, 46, 71 or 133, or at least three of positions 18, 23, 32, 46, 71 or 133, or at least four of positions 18, 23, 32, 46, 71 or 133, or at least five of positions 18, 23, 32, 46, 71 or 133, or at positions 18, 23, 32, 46, 71 or 133;

FGF-8 has been modified by replacement of the cysteine residues at position 10, 19, 109 or 127, or at least two of positions 10, 19, 109 or 127, or at least three of positions 10, 19, 109 and 127 with serine;

FGF-9 has been modified by replacement of the cysteine residue at position 68;

and

the position numbers are determined by reference to SEQ ID NO. 28 for FGF-5, SEQ ID NO. 30 for FGF-7, SEQ ID NO. 31 for FGF-8 and SEQ ID NO. 32 for FGF-9.

34. A plasmid, comprising the DNA fragment of any of claims 26-33.

35. The plasmid of claim 34 that is PZ2B1 and PZ2C1.

36. An E. coli cell transformed with a plasmid of claim 34.

37. A process for the production of a monogenous preparation of a cytotoxic conjugate in E. coli, comprising: culturing the cells of claim 36 under conditions whereby the cytotoxic conjugate is expressed; and isolating the cytotoxic conjugate.

38. A method of treating an FGF-mediated pathophysiological condition, comprising administering a therapeutically effective amount of the composition of claim 13.

39. A method of inhibiting proliferation of cells bearing FGF receptors, comprising contacting the cells with a proliferation inhibiting effective amount of a composition of claim 13.

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/US 94/08511

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48 C07K14/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,89 04832 (AMGEN INC.) 1 June 1989 see page 5 - page 6; claims 1-4	1,4-7, 17,26-36
X	EP,A,0 402 544 (FARMITALIA CARLO ERBA S.R.L.) 19 December 1990 see page 3, line 40 - line 44 see page 5, line 45 - line 52; claims	1-3,13, 14
X	WO,A,92 04918 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 2 April 1992 cited in the application See abstract, line 3	1-3

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Interna' l Application No
PCT/US 94/08511

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	CHEMICAL ABSTRACTS, vol. 117, no. 22, 30 November 1992, Columbus, Ohio, US; abstract no. 224962, see abstract & CIRC. RES., vol.71, no.3, 1992 pages 640 - 645 BIRO SADATOSHI ET AL. 'IN VITRO EFFECTS OF A RECOMBINANT TOXIN TARGETED TO THE FIBROBLAST GROWTH FACTOR RECEPTOR ON RAT VASCULAR SMOOTH MUSCLE AND ENDOTHELIAL CELLS'	1-3, 12-14, 38,39
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS), vol.269, no.17, 29 April 1994, BALTIMORE, MD US pages 12552 - 12558 DOUGLAS A. LAPPI ET AL. 'EXPRESSION AND ACTIVITIES OF A RECOMBINANT BASIC FIBROBLAST GROWTH FACTOR-SAPORIN FUSION PROTEIN.' see the whole document	1-3, 26-33
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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